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Application for United States Letters Patent

To all whom it may concern:

Be it known that we, Nicole Suciu-Foca, Zhuoro Liu, Chih-Chao Chang and Raffaello Cortesini

have invented certain new and useful improvements in

GENERATION OF ANTIGEN-SPECIFIC T SUPPRESSOR CELLS FOR TREATMENT OF REJECTION

of which the following is a full, clear and exact description.

GENERATION OF ANTIGEN SPECIFIC T SUPPRESSOR CELLS FOR TREATMENT OF REJECTION

This application is a continuation-in-part application of PCT International Application No. PCT/US00/16594, filed June 15, 2000, which claims priority and is a continuation-in-part application of U.S. Serial No. 09/333,809, filed June 15, 1999, the contents of which are hereby incorporated by reference into the present application.

This invention was made with support under Grant Nos. 5-RO1-A125210-11, RO1A125210-10, 5-RO1-A125210-12, and 5-RO1A123210-13 from the National Institutes of Health. Accordingly, the United States Government has certain rights in the invention.

Throughout this application, various references are referred to within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of this application, preceding the claims.

BACKGROUND OF THE INVENTION

Specific suppression of the host's immune response to donor HLA antigens remains the ultimate goal for clinical transplantation. In spite of considerable effort, however, allospecific human suppressor T cells (Ts) have been difficult to generate. The studies herein (first series of experiments) show that allospecific and xenospecific T_s can

be raised by multiple priming of human T cells in mixed lymphocyte cultures (MLC). T_s derive from the $CD8^+CD28^-$ subset and recognize specifically the MHC class I antigens expressed by Antigen-Presenting Cells (APC) used for in vitro immunization. Allospecific T_s prevent the upregulation of B7 molecules on target APCs, interfering with the CD28-B7 interaction required for T helper (T_h) activation. These findings provide a basis for the development of specific immunosuppressive therapy.

The induction of donor-specific tolerance remains the ultimate goal for clinical transplantation. Immunosuppressive treatments that have been developed so far act non-specifically, placing the recipient at increased risk for infections and malignancies.

Transplant tolerance has been induced in adult animals by inactivation or depletion of mature T lymphocytes prior to transplantation using cyclosporine (CsA) (1), total lymphoid irradiation (2,3), anti-lymphocyte serum (4), antibodies against CD4+ and CD8+ T cells (5), or donor-specific transfusions (6,7). Studies of peripheral graft tolerance have suggested the existence of an active mechanism of suppression which is donor-specific and can be transferred adoptively to secondary hosts (1, 7-10). However, there is still controversy concerning the phenotypic characteristics of these regulatory T cells and their MHC restriction, as both $CD8^+$ and $CD4^+$ T cells were reported to display suppressive activity (11). This controversy lead to the speculation that no distinctive T_s lineage actually exists. It has been suggested that suppression may result from antagonistic effects of

(Th)2-type lymphokines (such as IL-4 and IL-10) on the response of T_h1 cells (2,12), or from recognition by T_s of either idiotypic determinants of the TCR of alloreactive T cells or of MHC antigens expressed on stimulating cells (10,13). The generation of T_s lines has proven, however, to be a difficult task rendering the characterization of these cells hard to achieve.

The aim of the present study (first series of experiments) was to develop and characterize suppressor T cell lines which inhibit specifically the alloimmune response. This study established for the first time the existence of a population of $CD8^+CD28^-$ T_s which are allorestricted by HLA-class I antigens expressed by the cells used for priming. The mechanism of suppression is based on the capacity of T_s to prevent the upregulation of B7 molecules (CD80 and CD86) induced by Th on the stimulating APC. Allorestricted T_s can be easily and reproducibly expanded in cultures facilitating the <u>in vitro</u> study of immunoregulatory networks and the development of new strategies for specific immunosuppression.

Evidence that T cells can down-regulate the immune response by producing or consuming certain cytokines or by lysing APCs or T helper cells has been provided in various systems. However, the generation and characterization of suppressor T cell lines have met with limited success. In the second series of experiments herein it is shown that xenospecific suppressor T cells can be generated by <u>in vitro</u> stimulation of human T cells with pig APCs. Similar to allospecific suppressors, these xenospecific suppressor T cells carry the $CD8^+CD28^-$ phenotype and react to MHC class I antigens

expressed by the APCs used for priming. TCR spectratyping of T suppressor cells showed oligoclonal usage of TCR-Vβ families, indicating that xenostimulation of $CD8^+CD28^-$ T cells results in antigen-driven selection of a limited Vβ repertoire. Xenospecific T suppressor cells prevent the up-regulation of CD154 molecules on the membrane of T helper (Th) cells, inhibiting their ability to react against the immunizing MHC-class II xenoantigens. The mechanism of this suppression, therefore, appears to be blockade of CD154/CD40 interaction required for efficient costimulation of activated T cells.

The induction of regulatory T cells may offer an effective means for specific immunosuppression of autoimmune disease and allograft rejection. The existence of suppressor T cells has been previously documented, yet their mechanism of action remains poorly characterized. The third series of studies herein demonstrate that T suppressor (Ts) cell lines can be generated by <u>in vitro</u> immunization of human PBMCs, with synthetic peptides or soluble proteins coupled to beads. Such Ts cells express the $CD8^+CD28^-$ phenotype and show the following characteristics: a) antigen specificity and restriction by self MHC Class I molecules, b) limited TCR V beta gene usage, c) ability to inhibit antigen-specific, MHC Class II restricted, Th proliferative responses, and d) capacity to downregulate and/or inhibit the upregulation by Th of CD40, CD80, and CD86 molecules on APCs. The inhibitory activity of Ts on Th proliferation requires the tripartite interaction between Th, Ts, and APCs and results from inefficient costimulation of Th.

Understanding the mechanism which underlies the induction of

immunologic tolerance is crucial to the development of strategies for treatment of auto-immune diseases and allograft rejection. Although the concept that T suppressor cells (Ts) downregulate the immune response has long been accepted, the existence of a distinct population of lymphocytes that mediates suppression has not been convincingly demonstrated. In previous studies, human T cell lines (TCLs) were utilized to analyze the suppressive effects of $CD8^+$ $CD28^-$ T cells in allogeneic, peptide specific and xeno-specific responses. In each case, $CD8^+$ $CD28^-$ T cells inhibit proliferation of $CD4^+$ T helper lymphocytes (Th) with cognate antigen specificity. These $CD8^+$ $CD28^-$ T cells display the critical functional characteristics of T suppressor cells. Similar to the induction of $CD8^+$ cytotoxic T cells (Tc) by Th, this process depends on antigen presenting cells (APC) acting as a "bridge" between MHC-class I specific $CD8^+$ and class II specific $CD4^+$ T cells. A possible explanation of Ts-mediated suppression is their ability to modulate the function of APCs. The fourth series of studies herein show that $CD8^+CD28^-$ Ts directly inhibit the CD40 signaling pathway of APC by a contact-dependent mechanism that renders bridging APCs incapable of inducing $CD4^+$ The activation. The effects of Ts on the functional state of APC supports the concept that the order in which Ts and The cells interact with cognate APCs determines the functional outcome of immune responses.

SUMMARY OF THE INVENTION

This invention provides a method of generating antigen specific allospecific human suppressor CD8+CD28- T cells which comprises: a) obtaining peripheral blood T cells from a subject; b) stimulating by multiple priming a T cell line from the T cells obtained in step (a) with allogeneic antigen presenting cells (APCs), said APCs expressing an MHC class I antigen recognized by the primed T cell line and an MHC class II antigen recognized by CD4+ T helper cells from said primed T cell line; c) isolating primed CD8+ T cells and CD4+ T helper cells from the T cell line stimulated in step (b); d) isolating primed CD8+CD28- T cells from the isolated primed CD8+ T cells of step (c); e) detecting suppression by the primed CD8+CD28- T cells isolated in step (d) of interaction between the CD4+ T helper cells isolated in step (c) and allogeneic antigen presenting cells (APCs) expressing the same MHC class I antigen and the same MHC class II antigen expressed by the APCs used to stimulate the T cell line of step (b), thereby identifying antigen specific allospecific human suppressor CD8+CD28- T cells; and f) expanding the antigen specific allospecific human suppressor CD8+CD28- T cells identified in step (e), thereby generating the antigen specific allospecific human suppressor CD8+CD28- T cells.

This invention provides antigen specific allospecific human suppressor CD8+ CD28+ T cells produced by the method of generating antigen specific allospecific human suppressor CD8+CD28- T cells which comprises: a) obtaining peripheral blood T cells from a subject; b) stimulating by multiple priming a T cell line from the T cells obtained in step (a) with allogeneic antigen presenting cells (APCs), said APCs

expressing an MHC class I antigen recognized by the primed T cell line and an MHC class II antigen recognized by CD4+ T helper cells from said primed T cell line; c) isolating primed CD8+ T cells and CD4+ T helper cells from the T cell line stimulated in step (b); d) isolating primed CD8+CD28- T cells from the isolated primed CD8+ T cells of step (c); e) detecting suppression by the primed CD8+CD28- T cells isolated in step (d) of interaction between the CD4+ T helper cells isolated in step (c) and allogeneic antigen presenting cells (APCs) expressing the same MHC class I antigen and the same MHC class II antigen expressed by the APCs used to stimulate the T cell line of step (b), thereby identifying antigen specific allospecific human suppressor CD8+CD28- T cells; and f) expanding the antigen specific allospecific human suppressor CD8+CD28- T cells identified in step (e).

This invention provides a method of generating xenospecific human suppressor CD8+CD28- T cells which comprises: a) obtaining peripheral blood T cells from a human subject; b) stimulating by multiple priming a human T cell line from the T cells obtained in step (a) with xenogeneic mammalian antigen presenting cells (APCs), said APCs expressing a xenogeneic MHC class I antigen and a xenogeneic MHC class II antigen; c) isolating primed human CD8+ T cells and human CD4+ T helper cells from the T cell line stimulated in step (b); d) isolating primed human CD8+CD28- T cells from the isolated primed human CD8+ T cells of step (c); e) detecting suppression by the primed human CD8+CD28- T cells isolated in step (d) of interaction between the human CD4+ T helper cells isolated in step (c) and xenogeneic antigen presenting cells (APCs) expressing the same xenogeneic MHC class I

antigen and xenogeneic MHC class II antigen expressed by the xenogeneic APCs used to stimulate the human T cell line of step (b), thereby identifying xenospecific human suppressor CD8+CD28- T cells; f) expanding the xenospecific human suppressor CD8+CD28- T cells identified in step (e), thereby generating the xenospecific human suppressor CD8+CD28- T cells.

This invention provides xenospecific human suppressor CD8+ CD28+ T cells produced by the method of generating xenospecific human suppressor CD8+CD28- T cells which comprises: a) obtaining peripheral blood T cells from a human subject; b) stimulating by multiple priming a human T cell line from the T cells obtained in step (a) with xenogeneic mammalian antigen presenting cells (APCs), said APCs expressing a xenogeneic MHC class I antigen and a xenogeneic MHC class II antigen; c) isolating primed human CD8+ T cells and human CD4+ T helper cells from the T cell line stimulated in step (b); d) isolating primed human CD8+CD28- T cells from the isolated primed human CD8+ T cells of step (c); e) detecting suppression by the primed human CD8+CD28- T cells isolated in step (d) of interaction between the human CD4+ T helper cells isolated in step (c) and xenogeneic antigen presenting cells (APCs) expressing the same xenogeneic MHC class I antigen and xenogeneic MHC class II antigen expressed by the xenogeneic APCs used to stimulate the human T cell line of step (b), thereby identifying xenospecific human suppressor CD8+CD28- T cells; and f) expanding the xenospecific human suppressor CD8+CD28- T cells identified in step (e).

This invention provides a method of generating allopeptide

antigen specific human suppressor CD8+CD28- T cells which comprises: a) obtaining peripheral blood T cells from a subject; b) stimulating by multiple priming a T cell line from the T cells obtained in step (a) with autologous antigen presenting cells (APCs) pulsed with an allopeptide, said allopeptide comprising an amino acid sequence comprising both MHC class I and MHC class II amino acid sequences wherein the amino acid sequences are binding sequences (motifs) and are recognized by the primed T cell line; c) isolating primed CD8+ T cells and CD4+ T helper cells from the T cell line stimulated in step (b); d) isolating primed CD8+CD28- T cells from the isolated primed CD8+ T cells of step (c); e) detecting suppression by the primed CD8+CD28- T cells isolated in step (d) of interaction between the CD4+ T helper cells isolated in step (c) and autologous antigen presenting cells (APCs) expressing the same MHC class I and MHC class II binding motifs as expressed by the APCs used to stimulate the T cell line of step (b), thereby identifying allopeptide antigen specific human suppressor CD8+CD28- T cells; and f) expanding the allopeptide antigen specific human suppressor CD8+CD28- T cells identified in step (e), thereby generating the antigen specific human suppressor CD8+CD28- T cells.

This invention provides an antigen specific human suppressor CD8+CD28- T cells produced by the above-described method of generating the antigen specific human suppressor CD8+CD28- T cells.

This invention provides a method of determining whether a level of immunosuppresant therapy given to a subject undergoing the level immunosuppression therapy requires a

reduction which comprises: a) obtaining a blood sample from the subject; and b) determining the presence of T suppressor cells present in the sample, the presence of T suppressor cells indicating that the subject requires the reduction of immunosuppresant therapy.

This invention provides a method of reducing the risk of rejection of an allograft in a subject undergoing immunosuppression therapy which comprises: a) obtaining a blood sample from the subject; b) removing T suppressor cells from the blood sample; c) expanding the T suppressor cells of step (b); and d) reintroducing the expanded T suppressor cells of step (b) into the subject.

This invention provides a method of reducing the level of rejection of an allograft in a subject undergoing immunosuppression therapy which comprises administering to the subject the T suppressor cells produced by the above-described method of generating antigen specific allospecific human suppressor CD8+CD28- T cells, thereby preventing rejection of the tissue or organ transplant in the subject.

This invention provides a method of reducing the level of rejection of an allograft in a subject undergoing immunosuppression therapy which comprises administering to the subject the T suppressor cells produced by the above-described method of generating allopeptide antigen specific human suppressor CD8+CD28- T cells, thereby preventing rejection of the tissue or organ transplant in the subject.

This invention provides a method of preventing rejection of an allograft in a subject which comprises: a) obtaining a

blood sample from the subject; b) removing T suppressor cells from the blood sample; c) expanding the T suppressor cells of step (b); and d) reintroducing the expanded T suppressor cells of step (b) into the subject,
thereby preventing the rejection of the allograft in the subject.

This invention provides a method of preventing rejection of an allograft in a subject which comprises administering the T suppressor cells produced by the above-described method of generating antigen specific allospecific human suppressor CD8+CD28- T cells to the subject, thereby preventing rejection of the allograft in the subject.

This invention provides a method of preventing rejection of an allograft in a subject which comprises administering the T suppressor cells produced by the above-described method of generating allopeptide antigen specific human suppressor CD8+CD28- T cells to the subject, thereby preventing rejection of the allograft in the subject.

This invention provides a method of preventing rejection of a xenograft in a subject which comprises: a) obtaining a blood sample from the subject; b) removing T suppressor cells from the blood sample; c) expanding the T suppressor cells of step (b); and d) reintroducing the expanded T suppressor cells of step (b) into the subject,
thereby preventing the rejection of the xenograft in the subject.

This invention provides a method of preventing rejection of a xenograft in a subject which comprises administering the

T suppressor cells produced by the above-described method of generating xenospecific human suppressor CD8+CD28- T cells to the subject, thereby preventing rejection of the xenograft in the subject.

This invention provides a method of preventing autoimmune disease in a subject which comprises: a) obtaining a blood sample from the subject; b) removing T suppressor cells from the blood sample; c) expanding the T suppressor cells of step (b); and d) reintroducing the expanded T suppressor cells of step (b) into the subject,
thereby preventing autoimmune disease in the subject.

This invention provides a method of preventing autoimmune disease in a subject which comprises administering the T suppressor cells produced by the above-described method of generating antigen specific allospecific human suppressor CD8+CD28- T cells to the subject, thereby preventing autoimmune disease in the subject.

This invention provides a method of preventing autoimmune disease in a subject which comprises administering the T suppressor cells produced by above-described method of generating allopeptide antigen specific human suppressor CD8+CD28- T cells to the subject, thereby preventing autoimmune disease in the subject.

This invention provides a vaccine comprising allospecific T suppressor cells stimulated by APCs expressing an MHC class I antigen and an MHC class II antigen which T suppressor cells suppress an interaction between CD4+ T helper cells and allogeneic antigen presenting cells (APCs) expressing

the same MHC class I antigen and the same MHC class II antigen expressed by the APCs used to stimulate the allospecific T suppressor cells.

This invention provides a vaccine comprising xenospecific T suppressor cells stimulated by APCs expressing a xenogeneic MHC class I antigen and a xenogeneic MHC class II antigen which xenospecific T suppressor cells suppress an interaction between CD4+ T helper cells and xenogeneic antigen presenting cells (APCs) expressing the same xenogeneic MHC class I antigen and xenogeneic MHC class II antigen expressed by the APCs used to stimulate the xenospecific T suppressor cells.

This invention provides a method of inducing anergic T helper cells which comprises: a) incubating antigen presenting cells (APC) with allospecific T suppressor cells (Ts); b) overexpressing in the APC mRNA which encodes at least one monocyte inhibitory receptor (MIR), in a mixture of cells comprising the APCs from step (a), wherein overexpression of MIR transmits negative inhibitory signals to recruit an inhibitory signaling molecule, thyrosine phosphatase SHP-1 such that the APC are rendered tolerogenic; and c) incubating the APCs from step (b) with T helper cells (Th) to induce Th anergy.

This invention provides a method of generating a tolerogenic antigen presenting cell (APC) which comprises: a) contacting the APC with Ts; and b) overexpressing mRNA which encodes an MIR in the APC, thereby generating a tolerogenic antigen presenting cell (APC).

This invention provides a method of reducing the level of rejection of an allograft tissue or organ in a subject who is a transplant recipient of the allograft tissue or organ which comprises administering to the subject tolerogenic antigen presenting cells (APC) which overexpress monocyte inhibitory receptor (MIR), wherein the APC have been incubated with Ts prior to overexpression of MIR, thereby inducing Th anergy so as to prevent rejection of the tissue or organ allograft in the subject.

This invention provides a method of suppressing an autoimmune disease in a subject which comprises: a) contacting antigen presenting cells (APC) of the subject with T suppressor cells (Ts) specific for the antigen which induces the autoimmune disease; and b) administering to the subject the APC of step(a), thereby inducing tolerance to the antigen so as to suppress the autoimmune disease in the subject.

This invention provides a method of suppressing an autoimmune disease in a subject which comprises: a) overexpressing monocyte inhibitory receptor (MIR) in antigen presenting cells (APC) of the subject, which APC present the antigen which induces the autoimmune disease ; and b) administering to the subject the APC of step(a), thereby inducing tolerance to the antigen so as to suppress the autoimmune disease in the subject.

This invention provides a method of inducing tolerance to an allograft tissue or organ in a subject which comprises administering to the subject tolerogenic antigen presenting cells (APC) which overexpress monocyte inhibitory receptor

(MIR), thereby inducing tolerance to the allograft in the subject.

This invention provides a method of inducing tolerance to a xenograft tissue or organ in a subject which comprises administering to the subject tolerogenic antigen presenting cells (APC) which overexpress monocyte inhibitory receptor (MIR), thereby inducing tolerance to the xenograft in the subject.

This invention provides an antigen presenting cell (APC) which overexpresses ILT3, wherein the APC comprises a retroviral vector comprising a nucleic acid sequence which encodes ILT3 and ILT4 and overexpresses ILT3 and ILT4.

This invention provides a method of inducing tolerance to a xenograft tissue or organ transplant in a subject which comprises: a) introducing to an antigen presenting cell (APC) of a tissue or organ transplant donor a vector which overexpresses ILT3, wherein the vector comprises a nucleic acid sequence which encodes ILT3 and overexpresses ILT3; and b) administering the APC of step (a) to the subject, thereby inducing tolerance to the xenograft in the subject.

This invention provides a method of inducing tolerance to an allograft tissue or organ in a subject which comprises: a) introducing to an antigen presenting cell (APC) of a tissue or organ transplant donor a vector which overexpresses ILT3, wherein the vector comprises a nucleic acid sequence which encodes ILT3 and overexpresses ILT3; and b) administering the APC of step (a) to the subject, thereby inducing tolerance to the allograft in the subject.

This invention provides a method of treating an autoimmune disease in a subject which comprises: a) introducing to an antigen presenting cell (APC) of a subject having the autoimmune disease a vector which overexpresses ILT3, wherein the vector comprises a nucleic acid sequence which encodes ILT3 and overexpresses ILT3; and b) administering the APC of step (a) to the subject, thereby treating the autoimmune disease in the subject.

This invention provides a method of determining the appearance of T suppressor (Ts) cells which comprises detecting the level of expression of ILT3, ILT4 and ILT2 protein in APCs of a subject, wherein the subject is a xenograft tissue or organ transplant recipient which comprises: a) obtaining a sample from the subject; and b) detecting in the sample of step (a) overexpression of mRNA which encodes the ILT3, ILT4 and ILT2 protein in the APC of the subject, wherein detection of overexpression of mRNA which encodes the ILT3, ILT4 and ILT2 protein indicates the appearance of T suppressor cells in the subject.

This invention provides a method of determining the appearance of T suppressor (Ts) cells which comprises detecting the level of expression of ILT3, ILT4 and ILT2 protein in APCs of a subject, wherein the subject is an allograft tissue or organ transplant recipient which comprises: a) obtaining a sample from the subject; and b) detecting in the sample of step (a) overexpression of mRNA

which encodes the ILT3, ILT4 and ILT2 protein in the APC of the subject, wherein detection of overexpression of mRNA which encodes the ILT3, ILT4 and ILT2 protein indicates the appearance of T suppressor cells in the subject.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-1D. Detection and characterization of $CD8^+CD28^-$ Ts in alloreactive TCLs. Fig. 1A. Reactivity of unseparated TCL, separated $CD4^+$ and $CD8^+$ T cell subsets, and mixtures of $CD4^+$ and $CD8^+$ T cells to the specific allostimulator was determined in blastogenesis assays. Results are expressed as mean c.p.m. of triplicate reactions. The SD of the mean was less than 10%. Fig. 1B. Dose-dependent suppression of $CD4^+$ T cell alloreactivity to the specific stimulator in the presence of primed $CD8^+$, $CD8^+CD28^+$, and $CD8^+CD28^-$ T cells. Mean c.p.m. in cultures without T_s was 28,470±1730. Fig. 1C. Flow-cytometry determination of target cell apoptosis. The percent of allogeneic APCs stained by Annexin V was determined after 4 and 24 hours of incubation with or without $CD4^+$ and $CD8^+CD28^-$ T cells. Fig. 1D. Suppression of $CD4^+$ T cell proliferation to specific stimulator by $CD8^+$ $CD28^-$ T cells added to the cultures 0, 4, 8 and 16 hours after the initiation of the assay. Mean c.p.m. in cultures without T_s was 22,630±1860.

Figures 2A-2B. Antigenic specificity of $CD8^+CD28^-$ T suppressor cells. Fig. 2A. $CD4^+$ T_h from TCL SS-anti-JL were tested in blastogenesis assay for reactivity against APC from the original stimulator JL and from donors sharing with JL HLA-class I and class II (PO) or only class II (ST). Separate cultures were stimulated with a mixture of APCs from two donors (GC sharing with JL class I and ST sharing class II). $CD8^+CD28^-$ T_s from TCL SS-anti JL were added at the initiation of the blastogenesis assay. Percent suppression was calculated from the ratio of c.p.m. in cultures

containing mixtures of T_h and T_s and cultures containing only T_h. Mean c.p.m. in cultures without T_s was 33,212±2160, 27,630±1940, 28,430±2070 and 37,400±3450 when APCs from JL, PO, ST or mixtures of GC and ST were used as stimulators respectively. Fig. 2B. $CD4^+$ T_h from TCL SS-anti-JL and naive $CD4^+$ T cells from SS were stimulated with APC from JL. Alloreactive $CD8^+CD28^-$ T cells from SS generated by priming against JL, GC (sharing with JL class I) or ST (sharing with JL class II) were added to these cultures. Percent suppression was calculated as above.

Figure 3. Suppression of xenoreactivity by $CD8^+CD28^-$ T cells primed to xenogeneic cells. TCL was generated by priming human T cells against pig PBMC. Reactivity of unseparated TCL, separated $CD4^+$, $CD8^+$, $CD8^+CD28^-$ T cell subsets and mixtures of $CD4^+$ T cells with $CD8^+$ or $CD8^+CD28^-$ population to pig PBMC was determined in 3-day blastogenesis assay.

Figures 4A-4H. Expression of CD80 and CD86 on allostimulatory APC. $CD2^-$ depleted APCs from JL (the specific stimulator of TCL SS-anti-JL) were cultured for 24 hours without T cells (Figs. 4A and 4B), with $CD4^+$ T cells from SS-anti-JL (Figs. 4C and 4D), purified CD8+ CD28- T cells from SS anti-JL (Figs. 4E and 4F) and both $CD4^+$ and $CD8^+CD28^-$ T cells (Figs. 4G and 4H). Three-color flow cytometry was performed using mAbs anti-CD3 (for gating out the T cells), anti-CD80 and anti-CD86. Percent positive (%) and mean fluorescence intensity (MFI) of the positively staining population are indicated.

Figure 5. Prevention of suppression by mAb anti-CD28. $CD4^+$ and $CD8^+CD28^-$ T cells were separated from TCL SS-anti-JL and

tested alone or together for reactivity against APCs from JL in 3 day blastogenesis assays. MAbs anti-CD28 and anti-CTLA-4 (1μg/ml) were added to parallel cultures at the initiation of the assay. Percent suppression of CD4+ T cell proliferation induced by $CD8^+CD28^-$ T cells in the absence and in the presence of either mAb anti-CD28 or mAb anti-CTLA4 was calculated. Mean c.p.m. in T_h cultures without T_s were 32,510$\pm$2720.

Figures 6A-6B. Species specificity of $CD8^+CD28^-$ Ts cells. The response of alloreactive (SA-anti-BM) (Fig. 6A) and xenoreactive (SA-anti-pig A) (Fig. 6B) human T cell lines against the specific stimulator was measured in a 3-day proliferation assay. Reactivity of the unseparated TCLs, separated $CD4^+$ Th cells, and mixtures of $CD4^+$ and $CD8^+CD28^-$ (Ts) cells from either TCL is illustrated. Results are expressed as mean counts/min of triplicate reactions. The SD of the mean is indicated.

Figures 7A-7B. The suppressive effect of xenoreactive $CD8^+CD28^-$ T cells does not involve idiotypic or MHC-restricted interactions between Ts cells and Th cells. The reactivity of xenoreactive T cell lines from two human donors (MN and AP; Figs. 7A and 7B) against APCs from the same pig (pig B) was tested in a 3-day blastogenesis assay. The response of the unfractionated TCLs, purified $CD4^+$ cells, and mixtures of $CD4^+$ and $CD8^+CD28^-$ cells from both lines to pig APCs is presented as mean counts/min of triplicate reactions. The SD of the mean is indicated.

Figures 8A-8B. Fig.8A, Diffusion chamber experiments. Th

cells from TCL CG anti-W were tested for reactivity to APC from the specific stimulator (strain W) on close contact with Ts cells (from the same TCL) or separated from Ts cells by a semipermeable membrane. Percent suppression of Th cells reactivity by Ts cells is indicated. Fig.8B, Th cells from TCL ES-anti-W were tested for reactivity to APC from the specific stimulator (strain W) in the presence of autologous Ts cells and the indicated mAbs.

Figures 9A-9H. Cytokine profile of xenoreactive Th cells and Ts cells. Th cells and Ts cells from TCL CG-anti-pig Z were activated with PMA and ionomycin. Cells were treated with Brefeldin A, then fixed and stained with monoclonal antibodies specific for IL-2, IFN-γ, IL-4, and IL-10. Histograms obtained for the activated samples (solid line) and resting control samples (dotted lines) are presented. The results are representative of three independent experiments.

Figures 10A-10C. Failure of Ts cells to induce killing of pig APCs or human xenoreactive Th cells. Fig. 10A, Pig APCs were incubated for 4 hours with Th cells, Ts cells, or both Th and Ts cells. The percent of early apoptotic (annexin V positive, PI negative) and late apoptotic/necrotic (annexin V positive, PI positive) pig APCs in cultures with and without xenoreactive human T cells was determined by flow cytometry. Camptothecin-treated APCs were used as positive controls for apoptosis. Fig. 10B, $CD8^+$ $CD28^+$ and $CD8^+CD28^-$ T cells from TCL ES-anti-Q were tested for their ability to kill PHA-stimulated target cells from strain Q in a ^{51}Cr release assay. Results are expressed as percent lysis. Fig.

10C, Human Th cells were incubated with pig APCs in the presence or absence of Ts cells. The percent of $CD4^+$ human T cells undergoing apoptosis was determined by staining with annexin V and PI. The percent of early apoptotic (annexin V positive, PI negative) and late apoptotic/necrotic (annexin V positive, PI positive) Th cells is shown. $CD4^+$ Th cells treated with camptothecin served as positive controls.

Figures 11A-11D. Xenoreactive $CD8^+CD28^-$ T suppressor cells prevent up-regulation of CD40L expression on xenoreactive $CD4^+$ T cells. Human $CD4^+$ T cells were incubated for 6 hours without APCs (Fig. 11A), with APCs from the specific xenostimulator (pig W) (Fig. 11B), with APCs and Ts cells (Fig. 11C) or with control APCs from a pig (pig Z) that has different SLA class II antigens (Fig. 11D). CD154 expression on $CD3^+CD4^+$ human T cells was analyzed by flow cytometry. The percent CD154 positive T cells and the mean fluorescence intensity (MFI) are indicated. The results obtained with this TCL (CO-anti-pig W) are representative of data obtained from six TCLs.

Figure 12. Vβ repertoire of unstimulated $CD8^+CD28^-$ T cells from individual MN and of xenoreactive Ts cells from TCL MN-anti-pig B expressed as relative intensity. To analyze spectratypes, relative intensity was calculated as the peak area corresponding to each Vβ family divided by the sum of all peak areas.

Figures 13A-13D. Vβ repertoire of unstimulated $CD8^+CD28^-$ T cells from individual ES (Fig. 13A) and of Ts cells from TCL ES-anti-pig Q (Fig. 13B), ES-anti-pig W (Fig. 13C),

ES-anti-pig Z (Fig. 13D) expressed as relative intensity (histogram bars).

Figures 14A-14F. Vβ spectratyping of Ts cells from TCL MN-anti-pig B. Only the families with positive signal are shown. *x-axis*, fragment lengths on base pairs; *y-axis*, fluorescence amplitude.

Figures 15A-15C. Distribution of Jβ-Vβ combination fragments in the Vβ9 (Fig.15A), Vβ16 (Fig. 15B) and Vβ23 (Fig. 15C) families found in Ts cells from TCL ES-anti-pig Q. *x-axis*, fragment lengths on base pairs; *y-axis*, fluorescence amplitude.

Figure 16. Suppression of Th reactivity to rTT. $CD4^+$Th cells from a TT-specific TCL were tested in a 3-day blastogenesis assay for reactivity to rTT-pulsed APCs. $CD8^+CD28^-$ Ts from the same TCL were added to the cultures at the initiation of the proliferation assay.

Figure 17. Suppression of Th reactivity to peptide Tat-DR4. $CD4^+$ Th cells and $CD8^+$CD28- Ts were purified from a Tat-DR4-peptide specific TCL and tested for reactivity to the synthetic peptide in cultures containing irradiated APCs. $CD8^+CD28^-$ Ts were added to Th cultures at the initiation of the proliferation assay to measure suppressor activity.

Figures 18A-18B. Effect of anti-HLA class I mAbs on T cell reactivity to peptide Tat-DR4. $CD4^+$ Th were tested for reactivity to peptide Tat-DR4 in medium without or with mAbs specific for the HLA-class I antigens expressed by the

responder (Fig. 18A). Th and Ts were mixed together and tested for reactivity in the presence of MHC-class I specific mAbs (Fig. 18B).

Figures 19A-1-19-C-5. Vβ repertoire of $CD4^+$ Th and $CD8^+CD28^-$ Ts primed to rTT. The relative intensity of TCR Vβ families expressed by $CD8^+CD28^-$ Ts after five weeks (Figs. 19A-19E) and six weeks in cultures (Figs. 19F-19J) and by $CD4^+$ Th (Figs. 19K-19O) was calculated from the ratio between the peak area of each TCR and the sum of all area peaks.

Figures 20A-20L. $CD8^+CD28^-$ Ts prevent upregulation of CD40, CD80, and CD86 on antigen-loaded APCs. $CD4^+$ Th and $CD8^+CD28^-$ Ts from a Tat-DR4 peptide specific T cell line were incubated alone or together with autologous APCs and antigen for 24 hours. The expression of CD40, CD80, and CD86 on autologous APCs ($CD14^+$ and $CD20^+$ cells) was analyzed by flow-cytometry. The percent positive APCs and the mean fluorescence intensity (MFI) are indicated.

Figures 21A-21D. Ts mediated suppression of Th activation and proliferation requires the presence of APCs. CD4+ Th and CD8+CD28- Ts cells from the same TCL were activated alone or together either by mAb CD3 (Figs. 21A, 21B) or allogeneic APCs (Figs. 21C, 21D). Mouse IgG or autologous APCs were used as controls. Proliferation was determined in a three day assay (Figs. 21A, 21C), and CD40L expression on CD4+ T cells were analyzed after 6 h of culture (Figs. 21B, 21D). CPM of triplicate reactions are shown. SD to the mean was less than 10%. The results represent one of three independent experiments.

Figure 22. Early recognition of APCs by Ts is required for suppression of Th proliferation. CD4+ Th cells isolated from a TCL were cultured with APCs used for priming for 3 days. CD8+CD28- Ts cells from the same TCL were added 0, 6, or 18 h after initiation of the culture. Mean CPM of triplicate cultures are shown. SD was less than 10% of the mean. T cell reactivity to self APCs was less than 2,000 cpm. The data are from one of four independent experiments.

Figures 23A1-23E-5. Early recognition of APCs by Ts is required for suppression of the expression of costimulatory molecules on APCs. APCs used for stimulation were cultures alone (Fig. 23A-1-23A-5) or with allospecific Th for 48 h (Fig. 23B-1-23B-5). Ts were added at the initiation of the cultures (Fig. 23C-1-23C-5), after 6 h (Fig. 23D-1-23D-5) or after 18 h (Fig. 23E-1-23E-5). Expression of costimulatory molecules on CD14+ and CD20+ cells were analyzed at the end of the incubation period. Percent of positive cells is indicated. The data are the results of one of three repeat experiments.

Figure 24. Exogenous IL-2 restores Th reactivity in the presence of Ts. CD4+ Th and CD8+CD28- Ts cells from the same TCL were activated alone or together with allogeneic APCs. rIL-2 (% units/ml) was added to parallel cultures at the initiation of the blastogenesis assay. CPM of triplicate reactions are shown. SD to the mean was less than 10%. The resuklts represent one of thre repeat experiments.

Figure 25. Ts supress CD40-signaling in APC. The "suppressed" APC do not upregulate the expression of

costimulatory molecules (CD80, CD86) and are, therefore, unable to induce and sustain the full program of Th activity.

Figure 26. HLA A, B and DR values and split equivalence. Various HLA A loci, HLA B loci and HLA DR loci which may be used as antigens for priming T suppressor cells.

Figures 27A-27H. DRB Protein Sequences. Amino acid sequences of DRB proteins correspond to hypervariable regions of HLA-DR B1 antigens. These antigens may be used as allopeptides for priming T suppressor cells.

Figure 28. Detailed map of the swine major histocompatibility or swine leukocyte antigen (SLA) complex as compared to the human leukocyte antigen (HLA) complex. HLA-II ans SLA-II, HLA-II and SLA-III and Hla-I and SLA-I. (from J.K. Lunney and J.E. Butler, Immunogenetics, In The Genetics of the Pig, 1998, eds., M.F. Rothschild and A. Ruvinsky, CAB International.)

Figure 29. Amino acids sequences of SLA DRA alleles. These amino acid sequences may be used for generating xenospecific human suppressor T cells in the methods described infra.

Figure 30. Amino acids sequences of SLA DRB alleles. These amino acid sequences may be used for generating xenospecific human suppressor T cells in the methods described infra.

Figure 31. Amino acids sequences of SLA DQA alleles. These amino acid sequences may be used for generating xenospecific human suppressor T cells in the methods described infra.

Figure 32. Amino acids sequences of SLA DQB alleles. These amino acid sequences may be used for generating xenospecific human suppressor T cells in the methods described infra.

Figure 33. Nucleic acid sequences encoding ILT3 protein and the amino acid sequence of the encoded ILT3. (M. Cella and M. Colonna J. Exp. Med. 185, 1743 (1997))

Figures 34A-34B. Nucleic acid sequences encoding ILT4 protein and the amino acid sequences of the encoded ILT4. (M. Colonna et al. J. Immunol. 160, 3096 (1998))

Figure 35. Proliferation of CD8+CD28+ and CD8+CD28- cells from PBMC in response to allogeneic stimulation. PBMC from donor BB1 were labeled with CFSE and stimulated in MLC with CD2-depleted PBMC from donor BB2. Cells were labeled with CD28 PE and CD8 PerCP, and analyzed by flow cytometry at day 0, 5, and 7.

Figure 36. Proliferation of CD8+CD28+ and CD8+CD28- cells from allogeneic TCL. Responding CD8+ T cells from TCL BB1-anti-BB2 were stimulated with CD2-depleted PBMC from donor BB2 in the presence or absence of IL-2. Aliquots of these cultures were stained at time 0, 48, and 72 hr with CD28 PE, and analyzed by flow cytometry.

Figures 37A-37B. Suppression of alloreactivity of $CD4^+$ T cells by $CD8^+CD28^-$ T cells. Proliferative responses of $CD4^+$ Th cells from baboon (Fig. 37A) or rat (Fig. 37B) T cell lines against their specific stimulators were measured in a 3-day blastogenesis assay. Irradiated $CD8^+CD28^-$ Ts cells at

different Ts to Th ratios were added at the initiation of assay. The suppressive effect of Ts on Th was expressed as percent suppression.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides a method of generating antigen specific allospecific human suppressor CD8+CD28- T cells which comprises: a) obtaining peripheral blood T cells from a subject; b) stimulating by multiple priming a T cell line from the T cells obtained in step (a) with allogeneic antigen presenting cells (APCs), said APCs expressing an MHC class I antigen recognized by the primed T cell line and an MHC class II antigen recognized by CD4+ T helper cells from said primed T cell line; c) isolating primed CD8+ T cells and CD4+ T helper cells from the T cell line stimulated in step (b); d) isolating primed CD8+CD28- T cells from the isolated primed CD8+ T cells of step (c); e) detecting suppression by the primed CD8+CD28- T cells isolated in step (d) of interaction between the CD4+ T helper cells isolated in step (c) and allogeneic antigen presenting cells (APCs) expressing the same MHC class I antigen and the same MHC class II antigen expressed by the APCs used to stimulate the T cell line of step (b), thereby identifying antigen specific allospecific human suppressor CD8+CD28- T cells; and f) expanding the antigen specific allospecific human suppressor CD8+CD28- T cells identified in step (e), thereby generating the antigen specific allospecific human suppressor CD8+CD28- T cells.

Accordingly, the suppressor cells are obtained by a) isolating first the CD8+ T cells from the antigen-specific T cell lines; and b) isolating next the CD8+CD28- fraction from the CD8+ population above. Since only a small portion of suppressor T cells from step (d) is used for suppression analysis of step (e). Once suppression is detected in step

(e) the cells isolated in step (d) may be expanded by techniques known to the skilled artisan, for example by weekly restimulation of the APCs used to stimulate the T cells of step (b) in culture medium containing recombinant human interleukin-2 (IL-2).

In an embodiment of the above-described method of generating antigen specific allospecific human suppressor CD8+CD28- T cells the MHC class I antigen is an HLA-A or HLA-B antigen expressed by the APC used for priming. Antigen specific supressor cells can be generated by T cell priming against any of the existing HLA-A or HLA-B antigens of which there are more than two hundred such antigens. One of skill may select but is not limited to the HLA-A or HLA-B antigens from the group of HLA-A and HLA-B antigens listed in Figure 26.

In an embodiment of the above-described method of generating antigen specific allospecific human suppressor CD8+CD28- T cells the MHC class II antigen is an HLA antigen selected from the group consisting of HLA-DR, HLA-DQ and HLA-DP. One of skill in the art will recognize that there are hundreds of HLA class II antigens. For example HLA class II antigens may be but are not limited to DRB antigens which may be selected from but are not limited to the group of DRB proteins listed in Figure 27.

All APCs express two HLA-DR, HLA-DQ and two HLA-DP antigens. It is irrelevant which HLA-clas II antigens are expressed by the APCs, in order to generate suppressor T cells. It is important, however, that the response of CD4+ T helper cells to alloegeic APC can be inhibited only by CD8+ T suppressor

cells which recognize the MHC class I antigens expressed by the same APC.

This invention provides an antigen specific allospecific human suppressor CD8+ CD28+ T cells produced by the method of generating antigen specific allospecific human suppressor CD8+CD28- T cells which comprises: a) obtaining peripheral blood T cells from a subject; b) stimulating by multiple priming a T cell line from the T cells obtained in step (a) with allogeneic antigen presenting cells (APCs), said APCs expressing an MHC class I antigen recognized by the primed T cell line and an MHC class II antigen recognized by CD4+ T helper cells from said primed T cell line; c) isolating primed CD8+ T cells and CD4+ T helper cells from the T cell line stimulated in step (b); d) isolating primed CD8+CD28- T cells from the isolated primed CD8+ T cells of step (c); e) detecting suppression by the primed CD8+CD28- T cells isolated in step (d) of interaction between the CD4+ T helper cells isolated in step (c) and allogeneic antigen presenting cells (APCs) expressing the same MHC class I antigen and the same MHC class II antigen expressed by the APCs used to stimulate the T cell line of step (b), thereby identifying antigen specific allospecific human suppressor CD8+CD28- T cells; and f) expanding the antigen specific allospecific human suppressor CD8+CD28- T cells identified in step (e), thereby generating the antigen specific allospecific human suppressor CD8+CD28- T cells.

This invention provides a method of generating xenospecific human suppressor CD8+CD28- T cells which comprises: a) obtaining peripheral blood T cells from a human subject; b) stimulating by multiple priming a human T cell line from the

T cells obtained in step (a) with xenogeneic mammalian antigen presenting cells (APCs), said APCs expressing a xenogeneic MHC class I antigen and a xenogeneic MHC class II antigen; c) isolating primed human CD8+ T cells and human CD4+ T helper cells from the T cell line stimulated in step (b); d) isolating primed human CD8+CD28- T cells from the isolated primed human CD8+ T cells of step (c); e) detecting suppression by the primed human CD8+CD28- T cells isolated in step (d) of interaction between the human CD4+ T helper cells isolated in step (c) and xenogeneic antigen presenting cells (APCs) expressing the same xenogeneic MHC class I antigen and xenogeneic MHC class II antigen expressed by the xenogeneic APCs used to stimulate the human T cell line of step (b), thereby identifying xenospecific human suppressor CD8+CD28- T cells; f) expanding the xenospecific human suppressor CD8+CD28- T cells identified in step (e), thereby generating the xenospecific human suppressor CD8+CD28- T cells.

Expansion techniques which may be used to culture cells in step (f) are well known to the ordinary skilled artisan. For example the expansion technique described above for the method of generating antigen specific allospecific human suppressor CD8+CD28- T cells may be used to expand xenospecific human suppressor CD8+CD28- T cells. In an embodiment of the above-described method of generating antigen specific xenospecific human suppressor CD8+CD28- T cells the xenogeneic antigen presenting cells (APCs) may be mammalian antigen presenting cells (APCs). For example, the APCs may be pig APCs or primate APCs.

In an embodiment of the above-described method of

generating xenospecific human suppressor CD8+CD28- T cells, the xenogeneic mammalian antigen presenting cells (APCs) are selected from pig or primate APCs. One of skill in the art will recognize that pig antigens may be selected from numerous SLA antigens class I antigens. The antigens may be selected from but are not limited to the group of SLA DRA, SLA-DRB, SLA-DRQ-A and SLA-DQB listed in Figures 29 through 32.

In an embodiment of the above-described method of generating xenospecific human suppressor CD8+CD28- T cells, the xenogeneic MHC class I antigen is selected from the group consisting of swine histocompatibility leukocyte antigen (SLA) class-I and MHC class II antigen is selected from the group consisting of swine histocompatibility leukocyte antigen (SLA) class-II. T cells specific for any SLA class I antigen or class II MHC antigen may be obtained by using said SLA class I or II antigens for priming. The SLA antigens may be expressed by the APCs used for priming. APCs of other mammals including all species of primates may be used according to the above-described method.

This invention provides a xenospecific human suppressor CD8+ CD28+ T cells produced by the above-described method of generating xenospecific human suppressor CD8+CD28- T cells.

This invention provides a method of generating allopeptide antigen specific human suppressor CD8+CD28- T cells which comprises: a) obtaining peripheral blood T cells from a subject; b) stimulating by multiple priming a T cell line from the T cells obtained in step (a) with autologous antigen presenting cells (APCs) pulsed with an allopeptide,

said allopeptide comprising an amino acid sequence comprising both MHC class I and MHC class II amino acid sequences wherein the amino acid sequences are binding sequences (motifs) and are recognized by the primed T cell line; c) isolating primed CD8+ T cells and CD4+ T helper cells from the T cell line stimulated in step (b); d) isolating primed CD8+CD28- T cells from the isolated primed CD8+ T cells of step (c); e) detecting suppression by the primed CD8+CD28- T cells isolated in step (d) of interaction between the CD4+ T helper cells isolated in step (c) and autologous antigen presenting cells (APCs) expressing the same MHC class I and MHC class II binding motifs as expressed by the APCs used to stimulate the T cell line of step (b), thereby identifying allopeptide antigen specific human suppressor CD8+CD28- T cells; and f) expanding the allopeptide antigen specific human suppressor CD8+CD28- T cells identified in step (e), thereby generating the antigen specific human suppressor CD8+CD28- T cells. The identified human suppressor CD8+CD28- T cells are allospecific T suppressor cells. As discussed above any expansionmethod known to the skilled artisan may be used for expansion in culture of step (f).

In an embodiment of the above-described method of generating the antigen specific human suppressor CD8+CD28- T cells the allopeptide is a peptide antigen or a whole protein. For example the allopeptide may be selected from an allopeptide corresponding to hypervariable regions of HLA-DR B1 antigens which may be selected from but not limited to the HLA-DR B1 antigens listed in Figure 27.

This invention provides an antigen specific human suppressor

CD8+CD28- T cells produced by the above-described method of generating the antigen specific human suppressor CD8+CD28- T cells.

This invention provides a method of determining whether a level of immunosuppresant therapy given to a subject undergoing the level immunosuppression therapy requires a reduction which comprises: a) obtaining a blood sample from the subject; and b) determining the presence of T suppressor cells present in the sample, the presence of T suppressor cells indicating that the subject requires the reduction of immunosuppresant therapy.

The presence of T suppressor cells in the sample may be determined in step (b) as follows: CD4+ T cells and CD8+ T cells are isolated from the recipient's (the subject undergoing the level immunosuppression therapy) blood. The CD8+CD28- T cell subset is isolated from the CD8+ population of T cells. II. Cultures are set up as follows: I. Recipient CD4+ Th plus donor APCs (depleted of CD2+ cells); Recipient CD8+CD28- T cells plus donor APCs (depleted of CD2+ cells); Recipient CD4+ Th plus CD8+CD28- T cells plus donor APCs. In control cultures donor APCs are replaced by APCs from a subject with different HLA-class I antigens. III. APCs are stained with mAb specific for CD19 and CD14 (PE, and with mAb specific for CD80 (FITC). IV. Suppression is considered to be present when the level of CD80 expresion on donor APCs is 20% lower in cultures containing CD4+ Th and CD8+CD28- Ts than in cultures without Ts.

The presence of Ts in three consecutive samples of blood obtained at one month intervals indicates that the patient

(recipient, i.e. subject undergoing the level immunosuppression therapy) is "accepting" the graft (transplant) and that immunosuppression can be tapered down, i.e. decreased.

In an embodiment of the above-described method of determining whether a level of immunosuppresant therapy given to a subject undergoing the level immunosuppression therapy requires a reduction the T suppressor cells are suppressor CD8+CD28- T cells.

This invention provides a method of reducing the risk of rejection of an allograft in a subject undergoing immunosuppression therapy which comprises: a) obtaining a blood sample from the subject; b) removing T suppressor cells from the blood sample; c) expanding the T suppressor cells of step (b); and d) reintroducing the expanded T suppressor cells of step (b) into the subject.

In an embodiment of the above-desribed method of reducing the risk of rejection of an allograft in a subject undergoing immunosuppression therapy, the expansion in step (c) may be to produce an amount of approximately 10^7 T suppressor cells. One of skill is not limited to expanding the T suppresor cells to this amount of cells.

In another embodiment of the above-described method of reducing the risk of rejection of an allograft in a subject undergoing immunosuppression therapy the T suppressor cells are suppressor CD8+CD28- T cells.

This invention provides a method of reducing the level of rejection of an allograft in a subject undergoing immunosuppression therapy which comprises administering to

the subject the T suppressor cells produced by the above-described method of generating antigen specific allospecific human suppressor CD8+CD28- T cells, thereby preventing rejection of the tissue or organ transplant in the subject.

This invention provides a method of reducing the level of rejection of an allograft in a subject undergoing immunosuppression therapy which comprises administering to the subject the T suppressor cells produced by the above-described method of generating allopeptide antigen specific human suppressor CD8+CD28- T cells, thereby preventing rejection of the tissue or organ transplant in the subject.

This invention provides a method of preventing rejection of an allograft in a subject which comprises: a) obtaining a blood sample from the subject; b) removing T suppressor cells from the blood sample; c) expanding the T suppressor cells of step (b); and d) reintroducing the expanded T suppressor cells of step (b) into the subject,
thereby preventing the rejection of the allograft in the subject.

This invention provides a method of preventing rejection of an allograft in a subject which comprises administering the T suppressor cells produced by the above-described method of generating antigen specific allospecific human suppressor CD8+CD28- T cells to the subject, thereby preventing rejection of the allograft in the subject.

This invention provides a method of preventing rejection of an allograft in a subject which comprises administering the T suppressor cells produced by the above-described method of

generating allopeptide antigen specific human suppressor CD8+CD28- T cells to the subject, thereby preventing rejection of the allograft in the subject.

This invention provides a method of preventing rejection of a xenograft in a subject which comprises: a) obtaining a blood sample from the subject; b) removing T suppressor cells from the blood sample; c) expanding the T suppressor cells of step (b); and d) reintroducing the expanded T suppressor cells of step (b) into the subject,
thereby preventing the rejection of the xenograft in the subject.

In an embiodiment of the above-described method of preventing rejection of a xenograft in a subject the T suppressor cells are suppressor CD8+CD28- T cells. The suppressor CD8+CD28- T cells have to be primed with APCs from the donor, i.e. to specific xeno antigens before step (c), e.g. MHC class I antigens. CD8+CD28- T cells acquire T suppressor function only after priming with APCs expressing the donor's MHC class I antigens.

This invention provides a method of preventing rejection of a xenograft in a subject which comprises administering the T suppressor cells produced by the above-described method of generating xenospecific human suppressor CD8+CD28- T cells to the subject, thereby preventing rejection of the xenograft in the subject.

This invention provides a method of preventing autoimmune disease in a subject which comprises: a) obtaining a blood sample from the subject; b) removing T suppressor cells from

the blood sample; c) expanding the T suppressor cells of step (b); and d) reintroducing the expanded T suppressor cells of step (b) into the subject,
thereby preventing autoimmune disease in the subject.

In an embodiment of the above-decribed method of preventing autoimmune disease in a subject the expansion in step (c) may be to produce an amount of approximately 10^7 T suppressor cells. One of skill is not limited to expanding the T suppresor cells to this amount of cells.
In another embodiment of the above-decribed method of preventing autoimmune disease in a subject the T suppressor cells are suppressor CD8+CD28- T cells.

This invention provides a method of preventing autoimmune disease in a subject which comprises administering the T suppressor cells produced by the above-described method of generating antigen specific allospecific human suppressor CD8+CD28- T cells to the subject, thereby preventing autoimmune disease in the subject.

Suppressors CD8+CD28- T cells must be primed to acquire antigen specific function. The possible antigens which may be used for priming includes all peptides known to elicit an autoimmune disease

This invention provides a method of preventing autoimmune disease in a subject which comprises administering the T suppressor cells produced by above-described method of generating allopeptide antigen specific human suppressor CD8+CD28- T cells to the subject, thereby preventing autoimmune disease in the subject.

This invention provides a vaccine comprising allospecific T suppressor cells stimulated by APCs expressing an MHC class I antigen and an MHC class II antigen which T suppressor cells suppress an interaction between CD4+ T helper cells and allogeneic antigen presenting cells (APCs) expressing the same MHC class I antigen and the same MHC class II antigen expressed by the APCs used to stimulate the allospecific T suppressor cells.

In an embodiment of the above-described vaccine comprising allospecific T suppressor cells the APCs are allogeneic APCs said APCs expressing an MHC class I antigen recognized by the T suppressor cells and an MHC class II antigen recognized by allogeneic CD4+ T helper cells. In another embodiment of the above-described vaccine the APCs are APCs pulsed with an allopeptide, said allopeptide comprising an amino acid sequence having both MHC class I and MHC class II binding motifs wherein both motifs are recognized by the stimulated T suppressor cells. In an embodiment said allopeptide comprise an amino acid sequence comprising both MHC class I and MHC class II amino acid sequences wherein the amino acid sequences are binding sequences (motifs) and are recognized by the primed T cell line. In an embodiment of the above-described vaccine the T suppressor cells are suppressor CD8+CD28- T cells.

This invention provides a vaccine comprising xenospecific T suppressor cells stimulated by APCs expressing a xenogeneic MHC class I antigen and a xenogeneic MHC class II antigen which xenospecific T suppressor cells suppress an interaction between CD4+ T helper cells and xenogeneic antigen presenting cells (APCs) expressing the same

xenogeneic MHC class I antigen and xenogeneic MHC class II antigen expressed by the APCs used to stimulate the xenospecific T suppressor cells.

In an embodiment of the above-decribed vaccine comprising xenospecific T suppressor cells wherein the T suppressor cells are suppressor CD8+CD28- T cells.

This invention provides a method of inducing anergic T helper cells which comprises: a) incubating antigen presenting cells (APC) with allospecific T suppressor cells (Ts); b) overexpressing in the APC mRNA which encodes at least one monocyte inhibitory receptor (MIR), in a mixture of cells comprising the APCs from step (a), wherein overexpression of MIR transmits negative inhibitory signals to recruit an inhibitory signaling molecule, thyrosine phosphatase SHP-1 such that the APC are rendered tolerogenic; and c) incubating the APCs from step (b) with T helper cells (Th) to induce Th anergy.

In an embodiment of the above-described method, the monocyte inhibitory receptor (MIR) is selected from the group consisting of ILT4 (MIR-10), ILT2 (MIR7), and ILT3. In another embodiment of the above-described method, the Ts are allospecific human suppressor CD8+CD28- T cells. In a further embodiment of the above-described method, the Ts are xenospecific human suppressor CD8+CD28- T cells.
In yet another embodiment of the above-described method, the Ts allopeptide are antigen specific human suppressor CD8+CD28- T cells.

This invention provides a method of generating a tolerogenic

antigen presenting cell (APC) which comprises: a) contacting the APC with Ts; and b) overexpressing mRNA which encodes an MIR in the APC, thereby generating a tolerogenic antigen presenting cell (APC).

In an embodiment of the above-described method, the monocyte inhibitory receptor (MIR) is selected from the group consisting of ILT4 (MIR-10), ILT2 (MIR7), and ILT3. In another embodiment of the above-described method, the Ts are antigen specific allospecific human suppressor CD8+CD28- T cells. In a further embodiment of the above-described method, the Ts are xenospecific human suppressor CD8+CD28- T cells. In a still further embodiment of the above-described method, the Ts are allopeptide antigen specific human suppressor CD8+CD28- T cells.

This invention provides a method of reducing the level of rejection of an allograft tissue or organ in a subject who is a transplant recipient of the allograft tissue or organ which comprises administering to the subject tolerogenic antigen presenting cells (APC) which overexpress monocyte inhibitory receptor (MIR), wherein the APC have been incubated with Ts prior to overexpression of MIR, thereby inducing Th anergy so as to prevent rejection of the tissue or organ allograft in the subject.

In an embodiment of the above-described method, the monocyte inhibitory receptor (MIR) is selected from the group consisting of ILT4 (MIR-10), ILT2 (MIR7), and ILT3. In another embodiment of the above-described method, the Ts are allospecific human suppressor CD8+CD28- T cells. In yet another embodiment of the above-described method, the Ts are

xenospecific human suppressor CD8+CD28- T cells.
In a further embodiment of the above-described method, the Ts are allopeptide antigen specific human suppressor CD8+CD28- T cells.

This invention provides a method of suppressing an autoimmune disease in a subject which comprises: a) contacting antigen presenting cells (APC) of the subject with T suppressor cells (Ts) specific for the antigen which induces the autoimmune disease; and
b) administering to the subject the APC of step(a), thereby inducing tolerance to the antigen so as to suppress the autoimmune disease in the subject.

In an embodiment of the above-described method, the monocyte inhibitory receptor (MIR) is selected from the group consisting of ILT4 (MIR-10), ILT2 (MIR7), and ILT3. In another embodiment of the above-described method, the Ts are allospecific human suppressor CD8+CD28- T cells. In a further embodiment of the above-described method, the Ts are xenospecific human suppressor CD8+CD28- T cells.

In another embodiment of the above-described method, the Ts are allopeptide antigen specific human suppressor CD8+CD28- T cells.

This invention provides a method of suppressing an autoimmune disease in a subject which comprises: a) overexpressing monocyte inhibitory receptor (MIR) in antigen presenting cells (APC) of the subject, which APC present the antigen which induces the autoimmune disease and are genetically engineered to overexpress MIR; and b)

administering to the subject the APC of step(a), thereby inducing tolerance to the antigen so as to suppress the autoimmune disease in the subject.

In an embodiment of the above-described method, the monocyte inhibitory receptor (MIR) is selected from the group consisting of ILT4 (MIR-10), ILT2 (MIR7), and ILT3. In another embodiment of the above-described method, the Ts are allospecific human suppressor CD8+CD28- T cells. In still another embodiment of the above-described method, the Ts are xenospecific human suppressor CD8+CD28- T cells. In an embodiment of the above-described method, the Ts are allopeptide antigen specific human suppressor CD8+CD28- T cells.

This invention provides a method of inducing tolerance to an allograft tissue or organ in a subject which comprises administering to the subject tolerogenic antigen presenting cells (APC) which overexpress monocyte inhibitory receptor (MIR), thereby inducing tolerance to the allograft in the subject.

This invention provides a method of inducing tolerance to a xenograft tissue or organ in a subject which comprises administering to the subject tolerogenic antigen presenting cells (APC) which overexpress monocyte inhibitory receptor (MIR), thereby inducing tolerance to the xenograft in the subject.

This invention provides an antigen presenting cell (APC) which overexpresses ILT3, wherein the APC comprises a retroviral vector comprising a nucleic acid sequence which encodes ILT3 and overexpresses ILT3.

This invention provides an antigen presenting cell (APC) which overexpresses ILT3 and ILT4, wherein the APC comprises a retroviral vector comprising a nucleic acid sequence which encodes ILT3 and ILT4 and overexpresses ILT3 and ILT4.

In an embodiment of the above-described APCs, the APC is an APC from a subject who is a tissue or organ transplant donor.

This invention provides a method of inducing tolerance to a xenograft tissue or organ transplant in a subject which comprises: a) introducing to an antigen presenting cell (APC) of a tissue or organ transplant donor a vector which overexpresses ILT3, wherein the vector comprises a nucleic acid sequence which encodes ILT3 and overexpresses ILT3; and b) administering the APC of step (a) to the subject, thereby inducing tolerance to the xenograft in the subject.

This invention provides a method of inducing tolerance to a xenograft tissue or organ transplant in a subject which comprises: a) introducing to an antigen presenting cell (APC) of a tissue or organ transplant donor a vector which overexpresses ILT3 and ILT4, wherein the vector comprises a nucleic acid sequence which encodes ILT3 and ILT4 and overexpresses ILT3 and ILT4; and b) administering the APC of step (a) to the subject, thereby inducing tolerance to the

xenograft in the subject.

In an embodiment of the above-described methods the vector is a retroviral vector. Other suitable vectors can be used by one of skill in the above-descrobed method, wherein such vectors are able to infect hematopoietic cells at a high percentage.

This invention provides a method of inducing tolerance to an allograft tissue or organ in a subject which comprises: a) introducing to an antigen presenting cell (APC) of a tissue or organ transplant donor a vector which overexpresses ILT3, wherein the vector comprises a nucleic acid sequence which encodes ILT3 and overexpresses ILT3; and b) administering the APC of step (a) to the subject, thereby inducing tolerance to the allograft in the subject.

This invention provides a method of inducing tolerance to an allograft tissue or organ in a subject which comprises: a) introducing to an antigen presenting cell (APC) of a tissue or organ transplant donor a vector which overexpresses ILT3 and ILT4, wherein the vector comprises a nucleic acid sequence which encodes ILT3 and ILT4 and overexpresses ILT3 and ILT4; and b) administering the APC of step (a) to the subject, thereby inducing tolerance to the allograft in the subject. In an embodiment of the above-described methods the vector is a retroviral vector.

This invention provides a method of treating an autoimmune disease in a subject which comprises: a) introducing to an antigen presenting cell (APC) of a subject having the autoimmune disease a vector which overexpresses ILT3,

wherein the vector comprises a nucleic acid sequence which encodes ILT3 and overexpresses ILT3; and b) administering the APC of step (a) to the subject, thereby treating the autoimmune disease in the subject.

In an embodiment of the above-described method, the vector is a retroviral vector. In another embodiment the APCs which overexpress ILT3 present auto-antigenic peptides. In a preferred embodiment, the autoimmune disease is selected from, but is not limited to, the group consisting of diabetes, rheumatoid arthritis, and multiple sclerosis.

This invention provides a method of determining the appearance of T suppressor (Ts) cells which comprises detecting the level of expression of ILT3, ILT4 and ILT2 protein in APCs of a subject, wherein the subject is a xenograft tissue or organ transplant recipient which comprises: a) obtaining a sample from the subject; and b) detecting in the sample of step (a) overexpression of mRNA which encodes the ILT3 protein in the APC of the subject, wherein detection of overexpression of mRNA which encodes the ILT3 protein indicates the appearance of T suppressor cells in the subject.

In an embodiment of the above-described method expression of any one of or any combination of ILT3, ILT4 or ILT2 protein may be detected in APCs. In an embodiment of the above-described method the Ts are xenospecific human suppressor CD8+CD28- T cells.

This invention provides a method of determining the appearance of T suppressor (Ts) cells which comprises

detecting the level of expression of ILT3, ILT4 or ILT2 protein in APCs of a subject, wherein the subject is an allograft tissue or organ transplant recipient which comprises: a) obtaining a sample from the subject; and b) detecting in the sample of step (a) overexpression of mRNA which encodes the ILT3, ILT4 or ILT2 protein in the APC of the subject, wherein detection of overexpression of mRNA which encodes the ILT3,ILT4 or ILT2 protein indicates the appearance of T suppressor cells in the subject.

In an embodiment of the above-described methods the Ts are allospecific human suppressor CD8+CD28- T cells. In another embodiment of any of the above-described methods, the protein whose level of expression is detected may be any one of or any combination of ILT3, ILT4 or ILT2 protein. Such a method would also indicate the presence/appearance of Ts cells.

As used herein "appearance" of T suppressor (Ts) cells or "presence" thereof can be used interchangeably, i.e. detecting presence of Ts cells in a subject who is a transplant receipient (either xenograft or allograft) where no Ts cells of the type detected were present or had been previously present in an amount of much lower quantity than after the transplant.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

METHODS AND RESULTS

First Series of Experiments

Abbreviations used herein are: CsA - Cyclosporine; TCL - T cell line; Th - T helper cell; T_s - T suppressor cells TNF - tumor necrosis factor; PIN - Perforin-induced necrosis; PBMC - perpheral blood monoclear cells; PE - Phycoerythrin; MLR - Mixed lymphocyte reaction; APC - antigen-presenting cell.

METHODS

HLA Typing. Lymphocytes were typed for HLA class I and class II antigens by conventional serology. The class II genotype of the cells was determined by genomic typing of <u>in vitro</u> amplified DNA with sequence-specific oligonucleotide probes for DRB1, DQA1 and DQB1.

Generation of alloreactive T cell lines (TCL). Perpherial Blood mononuclear cells (PBMC) from healthy blood volunteers were separated from buffy coats by Ficoll-Hypaque centrifugation. Responding PBMCs ($1x10^6$/ml) were stimulated in 24-well plates with irradiated (1600 rad) APCs ($0.5x10^6$/ml) obtained from allogeneic PBMC by depletion of $CD2^+$ cells. Cells were co-cultured for 7 days in complete medium (RPMI 1640 supplemented with 10% human serum, 2mM L-glutamine and 50g/ml gentamicin) (GIBCO, NY). After 7 days responding cells were collected, washed and rechallenged with the original stimulating cells. Three days later rIL-2 (Boehringer Mannheim, IN) was added (10μg/ml) and the cultures were expanded for an additional 4 days.

Blastogenesis assays were performed on day 14. The HLA phenotypes of the responders and stimulators used for generating the seven different T cell lines used in these experiments are shown in **Table 1**. The xenoreactive TCL were generated by the same method using irradiated pig PBMC as stimulating cells.

Table 1 HLA Phenotypes of the Blood Donors

Donor ID	A	B	DR	DQA	DQB
TR	1,2	7,37	0701, 1501	0102, 0201	0201, 0602
RV	31, 33	35, 49	0701, 1501	0102, 0201	0201, 0602
NB	3, 24	27	0101, 0401	0101, 0301	0501, 0302
SS	26	35, 52	0402, 1502	0103, 0103	0302, 0601
JL	3, 29	44, 57	0701, 0701	0201, 0201	0201, 0201
GC	3, 74	45, 49	0801, 0801	0401, 0401	0402, 0402
ST	2, 24	13, 38	0701, 1301	0201, 0103	0201, 0603
PO	29, 31	44, 61	0701, 1101	0201, 0501	0201, 0301
LZ	2, 11	39, 67	1101, 1201	0501, 0501	0301, 0301

Cell separation and cultur . The $CD4^+$ and $CD8^+$ T cell subsets were isolated from PBMC by negative selection using Dynal $CD4^+$ and $CD8^+$ magnetic beads (Dynal, NY). Goat-anti-mouse Dynal beads were coupled with mAb anti-CD28 (Becton Dickinson, CA) according to the manufacturer's instructions. To separate $CD28^+$ and $CD28^-$ T cells from $CD8^+$ T cell suspensions, isolated $CD8^+$ T cells ($1x10^7$/ml) were incubated with $4x10^7$ CD28 beads for 20 min at 4°C. The suspension was then placed on a magnetic particle concentrator for 2-3 minutes. The unbound cells ($CD8^+CD28^-$) were transferred to another tube and washed three times in complete medium. The bound $CD8^+CD28^+$ T cell population was detached from the beads by overnight incubation at 37°C. Cells were collected, washed and resuspended in complete culture medium. Cytofluorographic analysis showed that the purity of $CD4^+$, $CD8^+$ and $CD8^+CD28^+$ suspensions was >96%. The $CD8^+CD28^-$ population contained less than 7% $CD28^+$ T cells.

Flow Cytometry. T cell subsets were defined using mAb CD4-PerCP, CD8-FITC, CD28-phycoerythrin from Becton Dickinson Immunocytometry System, CA. Cell suspensions were phenotyped prior to use in blastogenesis assays using a FACScan flow cytometer instrument (BDIS) equipped with a 15mm Argon Laser. To study the effect of T_h and T_s on the expression of B7 molecules on allogeneic APC, $CD4^+$ and $CD8^+$ T cells were isolated from alloreactive TCL by positive selection using CD4 and CD8 magnetic beads. The $CD8^+CD28^-$ subset was obtained from the CD8 population by negative selection using beads coupled with anti-CD28 mAB. T_h cells were cultured with APC from the specific stimulator at a 5:1 ratio. T_s were cultured with the APC at a 1:1 ratio. In mixed cultures containing T_h, T_s, and allogeneic APC the

ratio was 5:1:1. APC to which no T cells were added served as a control. After 24 hours of incubation cells were stained with saturating amounts of mAbs recognizing CD3-PerCP, CD80-PE (Becton Dickinson) and CD86-FITC (Pharmingen, CA). $CD3^+$ T cells were gated out, and the remaining cells were analysed using CellQuest software on a 650 Apple Macintosh Computer. Five parameter analysis (forward scatter, side scatter and 3 fluorescence channels) were used for list mode data analysis. FL3 channel was used as fluorescence trigger, FL1 and FL2 as analysis parameters. Mouse IgG ($\gamma 1$ and $\gamma 2$) reagents were used as isotype controls for non-specific binding of test reagents and as markers for delineating the positive and negative populations. CaliBrite flow cytometer beads (Becton Dickinson) and FACSComp program were used for calibration of the cytometer.

Flow Cytometry Analysis of Apoptosis. The capacity of alloreactive $CD8^+CD28^-$ T cells to induce apoptosis of CD2-depleted allogeneic APC was tested by flow cytometry using annexin V as a marker for apoptotic cells. APCs were incubated for 4 hrs or 24 hrs at 37° with T_h, T_s, and mixtures of T_h and T_s. The ratio between T cells and APC was 5:1. Cells were stained with mABs recognizing CD20-PE or CD4-PE (Becton Dickinson). After 15 min. of incubation cells were washed and stained with annexin V-FITC and propidium iodide according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Log FL2 (PE) versus Side scatter parameters were used to gate on $CD20^+$ or $CD4^+$ cells. Log FL1 (FITC) versus FL3 (PI) dot plot of the gated cells were used for cell apoptosis and necrosis analysis.

Proliferation Assays. Unseperated responding T cells were tested for blastogenesis at a concentration of $5x10^4$/well. T cell subsets sorted from the same cultures ($CD4^+$ T, $CD8^+$ T, $CD8^+CD28^+$ T, or $CD8^+CD28^-$ T) were tested at a concentration that corresponded to their frequency in the unseperated population as determined by flow cytometry analysis of the TCL and of each population sorted. In experiments which required no comparison between unseperated TCL and T cell subsets, CD4+ and CD8+ T were tested at $2.5x10^4$ cells/well and $CD8^+CD28^-$ T cells were tested at $1.25x10^4$ cells/well. Cell concentrations were adjusted after establishing the purity of the fraction by flow cytometry. In all blastogenesis assays the concentration of APC used for stimulation was $5x10^4$/well. After 48 hr of incubation, the cultures were pulsed with [^{3}H] Thymidine and harvested 18 hr later. [^{3}H] Thymidine incorporation was determined by scintillation spectrometry in an LK Betaplate counter. Mean cpm of the triplicate cultures and the standard deviation to the mean were calculated.

The percent suppression was calculated as follows:
% suppression = [1- (c.p.m. in mixed cultures of activated $CD4^+$ T_h and $CD8^+CD28^-$ T_s)/(c.p.m. in cultures with activated $CD4^+$ T_h)] x 100

The effect of mAb anti-CD28 (clone 37407.11 from R & D Systems, Minneapolis, Mn) and anti-CTLA4 (clone BN 13 from Coulter, Miami, Fl) on the suppressor effect was tested by adding the mAb to the cultures (1μg/ml) at the initiation of the blastogenesis assay.

Statistical Analysis. Statistical analysis of the results

was performed using BMDP statistical software. Analysis of variance to assess significance of group differences (ANOVA) followed by Tukey's method for multiple comparison was applied to flow cytometry results. Correlation coefficients were obtained using Linear Regression Analysis.

Results

Suppression of alloreactivity is mediated by $CD8^+CD28^-$ T cells and is dose-dependent. TCL were generated by stimulating PBMC from HLA unrelated individuals with irradiated APCs from blood donors mismatched from the responders for both HLA-class I and class II antigen. The HLA phenotypes of the blood donors used in this study are shown in **Table 1**. Although all TCL showed strong reactivity after primary and secondary stimulation, they displayed low reactivity when challenged for the third time in 3-day blastogenesis assays.

However, strong reactivity of $CD4^+$ T cells to the specific stimulator was restored when $CD8^+$ T cells were depleted from the TCL. $CD8^+$ T cells from the same culture showed little blastogenic response against the stimulator. When the cultures were reconstituted by mixing together the $CD4^+$ and $CD8^+$ subsets of cells, at the original ratio, the response of $CD4^+$ Th to the allogeneic priming cells was inhibited (**Fig. 1A**). The percent inhibition by $CD8^+$ T_s from different TCL ranged from 50 to >90% (mean 76%±23%). These experiments indicate that the $CD8^+$ fraction of the TCL contains a population of Ts which suppress the proliferative response of $CD4^+$ T_h against the specific stimulator.

To characterize the population of $CD8^+$ T cells which mediate suppression, the $CD8^+CD28^+$ and $CD8^+CD28^-$ populations from an alloreactive TCL (TCL-LZ anti-NB) were separated and tested for their capacity to inhibit the proliferative response of $CD4^+$ T cells from the same TCL against the specific stimulator (NB). The $CD8^+$ $CD28^-$ T cells exhibited dose-dependent suppression of the $CD4^+$ T cell response to the specific allostimulator, while the $CD8^+CD28^+$ T cells had no inhibitory effect (**Fig. 1B**). Naive $CD8^+$ T cells from the peripheral blood of the same responder had no inhibitory effect on the reactivity of CD4 T cells from this TCL (data not shown).

Since the kinetics of proliferative responses can shift under certain culture conditions the proliferative response of Th cells to allogeneic APC was measured in the presence or absence of T_s cells, by harvesting the cultures after 24, 48, 72, 96, and 120 hours of incubation. The data in **Table 2** show that suppression of Th proliferation became detectable after 48 hours. The magnitude of the suppressor effect increased over the next 3 days of incubation. Because the peak of Th blastogenesis occured on day 3 all suppressor assays were harvested after 72 hours of incubation.

To establish whether killing of stimulating cells by allo-activated CD8+ T cells contributes to the suppressive effect, the ability of separated $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells to lyse PHA-activated target cells from the specific stimulator was tested. ^{51}Cr release assays showed that the cytotoxic activity resided entirely in the $CD8^+$ $CD28^+$ T cell population (data not shown).

Since lymphocyte-mediated cytoxicity maybe caused not only by perforin induced necrosis (PIN) of the target but also by PIN/granzyme induced apoptosis (14) the capacity of T_s to cause apoptosis of allogeneic target cells was tested. No evidence of T_s -induced apoptosis was found. The percent apoptotic APC was similar in cultures to which Ts cells were added and in cultures containing only APC or T_h and APC (**Fig. 1C**). Also T_s did not cause apoptosis of T_h as the percentage of annexin V positive T_h was the same in cultures with or without T_s (data not shown). Hence the suppressive activity exhibited by allostimulated $CD8^+CD28^-$ T cells on the proliferative response of $CD4^+$ T cells is not caused by killing of the stimulating cell targets or by killing of Th cells.

Table 2

Kinetics of the responses of T_h and T_s cells

Incubation time (h)	(^{3}H) Thymidine incorporation (mean c.p.m. ± SD)					
	T_h		T_s		$T_h + T_s$	
	LZ	NB	LZ	NB	LZ	NB
24	14,190 ± 986	17,840 ± 1532	2315 ± 204	3796 ± 284	13,806 ± 1067	16,396 ± 1353
48	3081 ± 274	31,783 ± 2654	454 ± 29	3529 ± 310	4,143 ± 342	16,878 ± 1625
72	599 ± 57	31,673 ± 2015	92 ± 8	970 ± 67	491 ± 42	7606 ± 548
96	1610 ± 135	23,372 ± 1763	57 ± 4	155 ± 14	162 ± 12	780 ± 67
120	145 ± 14	15,940 ± 1032	50 ± 3	84 ± 7	96 ± 7	820 ± 75

$CD4^+$ T_h cells from TCL (LZ anti-NB) were tested for reactivity to specific target cells in the absence or presence of $CD8^+CD28^-$ T_s cells derived from the same TCL. The cultures were harvested at 24, 48, 72, 96, and 120h after initiation.

Suppression of CD4$^+$ T cell alloreactivity by allostimulated CD8$^+$CD28$^-$ T cells is an early event. To investigate the kinetics of the suppressive effect, CD8$^+$ CD28$^-$ T cells were isolated from an alloreactive TCL (TR anti-NB) and added to CD4$^+$ T cells from the same TCL at the initiation of the blastogenesis assay or after 4, 8 or 16 hours. Results obtained on day 3 showed that suppression was highest (90%) when CD8$^+$CD28$^-$ T cells were added at the initiation of the cultures (**Fig. 1D**). When T_s were added 4 or 8 hours after exposure of CD4$^+$ T cells to the specific stimulator, the inhibitory activity decreased to 50 and 20 percent, respectively. No inhibitory effect was observed when the addition of Ts was delayed by 16 hours after activation of CD4$^+$ T cells. The correlation between the time when Ts were added and their inhibitory effect was statistically significant ($r= 0.953$, $p< 0.05$). This indicates that suppression of specific reactivity of T_h cells by activated CD8$^+$CD28$^-$ T cells is an early event.

In an attempt to determine which surface molecule(s) and lymphokines may play a role in suppression we performed blocking experiments in which various mAbs were added to the cultures at the initiation of the blastogenesis assay. Antibodies to IL-4, IL-10 and (TNF)-β did not block the suppressive activity of CD8$^+$CD28$^-$ T cells, indicating that these cytokines are not the mediators of the inhibitory effect. The addition of anti-HLA-class I mAb to the cultures,

however, reduced the amount of suppression, indicating that allorecognition of HLA class I antigens by Ts is required for suppression to occur (data not shown).

Antigenic specificity of alloactivated $CD8^+CD28^-$ T_s. To determine the nature of the HLA-antigens recognized by $CD8^+CD28^-$ T_s, APC sharing with the original priming cells HLA-class II (required for responder $CD4^+$ T cell activation), or both HLA class I and class II antigens were used as stimulators in suppressors assays. One of the six representative experiments is described below.

Ts obtained from TCL SS-anti-JL inhibited by 51% the response of separated $CD4^+$ T cells to the original stimulator (JL). Reactivity against other APC (PO) sharing both HLA-class I and class II antigens was equally suppressed, while the response to APC (ST) sharing only class II antigens with the specific stimulator was not affected (**Fig. 2A**). Hence, $CD8^+CD28^-$ T_s recognize specifically HLA-class I antigens expressed by the APC used for <u>in vitro</u> immunization.

To establish whether APCs must co-express the target antigens recognized by $CD4^+$ Th and $CD8^+CD28^-$ Ts for suppression to occur, mixtures of two APC, sharing with the original priming cells either class II (ST) or class I (GC) antigens (but not both) were used for stimulation. There was no inhibition of $CD4^+$ Th reactivity by $CD8^+$ $CD28^-$ T_s in these blastogenesis assays, indicating that suppression requires cell-to-cell interaction between $CD4^+$ T_h, $CD8^+$ Ts and allogeneic APC expressing the class I and class II antigens against which the T cells were primed (**Fig. 2A**).

To further substantiate the conclusion that $CD8^+CD28^-$ Ts recognize HLA-class I antigens on allogeneic APC, $CD4^+$ T cells from TCL SS-anti-JL were mixed with autologous $CD8^+CD28^-$ Ts from other TCLs which had been primed against APC sharing with the original stimulator (JL) either HLA-class I (GC) or class-II (ST) antigens (Fig. 2b). Suppression was induced by $CD8^+CD28^-$ T cells (TCL SS-anti-GC) primed against shared HLA-class I antigen(s) (GC). $CD8^+CD28^-$ T cells from a TCL (TCL SS anti-ST) primed against shared HLA-DR, but different class I alloantigens (ST), had no suppressive effect. Hence, Ts generated by allostimulation are allorestricted by HLA-class I antigens.

To determine whether $CD8^+CD28^-$ T_s can also inhibit the recognition phase of the MLC response and whether such an effect is allo-MHC-class I restricted, alloactivated $CD8^+CD28^-$ T cells were added to a primary MLC at the initiation of the cultures. For this MLC, naive $CD4^+$ T cells obtained from peripheral blood (SS) were tested as responders against one stimulator (JL). Ts from the three TCL used in the experiment described above were tested for inhibitory activity. Only Ts which had been activated against the allogeneic stimulator used for the primary MLC (JL) or against APC sharing with JL an HLA-class I antigen (GC) were able to inhibit the primary MLC. $CD8^+CD28^-$ T cells from a TCL primed against stimulating cells sharing with JL HLA-class II but not class I antigens (ST) had no suppressor effect (**Fig. 2B**). These data indicate that alloactivated $CD8^+CD28^-$ T cells which recognize HLA-class I antigens specifically suppress the activation of $CD4^+$ T cells via the

direct recognition pathway.

Lack of MHC restriction of T_h-T_s interaction. The interaction between alloreactive $CD4^+$ T_h and $CD8^+CD28^-$ T_s may require T cell recognition of peptide(s) presented by self HLA-class I or class II antigens expressed by the responding or suppressing T cell population. To examine this possibility we generated alloreactive TCLs by priming T cells from three individuals (TR, RV and LZ) against the same allogeneic APC (NB). These three responders shared with each other either class I or class II antigens: RV shared with TR HLA-DR and DQ but not HLA-A, B; LZ shared with TR only HLA-A2. $CD4^+$ T cells from one of these TCLs (TR anti-NB) were next tested in 3 day blastogenesis assays for reactivity against APC from the specific stimulator. The proliferative response of $CD4^+$ T cells was inhibited with equal efficiency by autologous and allogeneic CD8+CD28- T cells which had been primed to the same stimulator. Thus, the regulatory effect of $CD8^+CD28^-$ T cells on $CD4^+$ responding T cells is not restricted either by the HLA-class I or class II antigens which they express (**Table 3**).

Suppression of xenoreactivity by $CD8^+$ $CD28^-$ T cells. The finding that the direct recognition pathway of alloreactivity can be suppressed specifically by activated $CD8^+CD28^-$ T cells has important implications for specific inhibition of allograft immunity. To determine whether <u>in vitro</u> educated T cells can also inhibit direct recognition of xenogeneic target cells, we generated xenoreactive TCLs by priming human PBMC with irradiated PBMC from a pig. Responding cells were primed on day 0 and restimulated on day 7 with pig PBMC. IL-2 was added on day 10, and the

cultures were tested on day 14 for reactivity against pig PBMC. While the non-fractionated TCL showed low blastogenic responses, the purified CD4$^+$ subset showed strong reactivity to pig stimulating cells in 3-day blastogenesis assays. Neither unseparated CD8$^+$ T cells or separated CD8$^+$CD28$^-$ T cells proliferated in response to the priming cells. When added to CD4$^+$ responding cells at the beginning of the assay both CD8$^+$ and CD8$^+$CD28$^-$ T cells strongly inhibited the proliferative response (**Fig. 3**). These results were confirmed in a large series of experiments. Experiments using inbred strains of swine showed that T_s were xenorestricted by MHC-class I antigens (see Third Series of Experiments).

Table 3

Lack of MHC Restriction of T Helper-Suppressor Cell Interactions

$CD4^+$ T responding cells	CD+CD28-T_s	[^{3}H] Thymidine Incorporation (mean cpm) Stimulators	
		TR	NB
TCL TR anti-NB		1,159±80	24,454±1960
TCL TR anti-NB	TCL TR anti-NB	689±50	611±40
TCL TR anti-NB	TCL RV[a] anti-NB	4,199±370	1,179±90
TCL TR anti-NB	TCL LZ[b] anti-NB	4,271±280	785±80

[a]Sharing with TR HLA-DR, and DQ but not HLA-A and B.
[b]Sharing with TR only HLA-A2.
$CD4^+$ T cells from an alloreactive TCL (TR anti-NB) were tested for specific alloreactivity in the presence of $CD8^+$ $CD28^-$ T cells from the same TCL or from other TCLs which have been generated by priming PBMC from unrelated blood donors against the same allogeneic stimulator.

These data indicate that human $CD8^+$ $CD28^-$ T cells can be educated <u>in vitro</u> to suppress the response of $CD4^+$ T cells against xenogeneic target cells.

Expression of CD80 and CD86 on stimulatory APC in the presence of T_s. Since inhibition of alloreactivity occured only when the stimulating APCs were recognized by both T_s and T_h, we examined the possibility that T_s interfere with the expression of costimulatory molecules. Interaction of CD28 on T cells with B7 molecules (CD80 and CD86) on APCs is the most important costimulatory pathway for the response to alloantigens (15,16). CD80 and 86 are absent on resting B and T cells, but are induced after activation. While CD86 is constitutively present on resting monocytes, CD80 is expressed only after stimulation with IFN-γ (17). To determine whether T_s alter the pattern of B7 expression on allogeneic APC, we cultured T_h and/or T_s from five different individuals with CD2-depleted APC from the specific allogeneic stimulator. Figure 4 illustrates the results of one of these experiments. Analysis of CD80 and CD86 expression on stimulating APC, showed that after 24 hours the level of expression increased significantly in the presence of alloreactive $CD4^+$ Th (Figs. 4C and 4D) compared with the level of expression in the absence of T cells (Figs. 4A and 4B) or in the presence of $CD8^+CD28^-$ T_s (Figs. 4E and 4F). APC from parallel cultures containing both T_h and T_s displayed much lower levels of CD80 and CD86 expression (Figs. 4G and 4H) than APC cultured only with T_h. This indicates that allospecific $CD8^+CD28^-$ T_s interfere with the upregulation of CD80 and CD86 expression induced on stimulating APCs by alloreactive $CD4^+$ T_h.

To analyze the statistical significance of the decreased B7 expression in cultures containing T_s, the results obtained in the five different experiments were grouped together. Comparison of the mean percentage of target cells expressing B7 molecules in cultures containing only T_h and in cultures with both T_h and T_s showed that the decrease in the upregulation of B7 expression in the presence of T_s was statistically significant (Table 4).

The impaired upregulation of CD80 and CD86 on stimulating APC may prevent the efficient costimulation of T_h in the presence of T_s. Alternatively, this may be the consequence rather than the cause of T_h inhibition by T_s. To explore these possibilities we tested the effects of mAbs anti-CD28 and anti-CTLA-4 on the reactivity of $CD4^+$ T_h from a TCL (SS anti-JL) to the specific stimulator (JL) in the presence and absence of $CD8^+CD28^-$ Ts. MAb anti-CTLA-4 did not affect T_s-mediated inhibition of the Th proliferative response. In contrast, ligation of CD28 by use of mAb anti-CD28 restored the ability of $CD4^+$ T_h to respond to the specific stimulator in the presence of $CD8^+CD28^-$ T_s (Fig. 5). This result is consistent with the notion that suppression is caused by defective costimulation.

Table 4

Effect of T_s cells on Th induced up-regulation of B7 molecules on APC

	Percentage of $B7^+$ target cells				
	APC	T_h+T_s+APC	T_h+APC	T_s+APC	P value[a]
CD80	12.4 ± 4.6[b]	20.4 ± 4.0[b]	47.2 ± 11.8	13.0±3.8[b]	0.0001
CD86	18.6 ± 12.4[b]	35.8 ± 15.5[c]	66.4 ± 7.1	27.6 ± 19.2[b]	0.0005

Results are expressed as mean ± SD of five different experiments.
[a] *P* value for differences between all groups was computed by ANOVA.
[b] The difference between B7 expression on targets incubated with T_h versus targets incubated with T_s, T_s plus T_h or medium was significant ($P < 0.01$).
[c] The difference between CD86 expression on targets incubated with T_h only versus targets cultured with both T_h and T_s was significant ($P < 0.05$).

DISCUSSION

The present study demonstrates that allospecific and xenospecific human Ts can be generated and expanded in vitro by multiple priming of PBMCs with allogeneic or xenogeneic stimulator cells. Alloreactive Ts derive from the $CD8^+CD28^-$ population of T lymphocytes and recognize specifically the MHC-class I antigens expressed by the allogeneic APC used for in vitro immunization. Suppression of Th alloreactivity occured only when the stimulatory APC co-expressed MHC class II antigens recognized by the Th to be suppressed and MHC-class I antigens recognized by the suppressor T cell population. Thus, $CD4^+$ T_h reactivity was not inhibited by $CD8^+$ $CD28^-$ T_s when the cells were stimulated in blastogenesis assay with mixtures of two allogeneic APCs sharing with the original priming cells either MHC class I or class II antigens. Suppression of alloreactive T_h, therefore, requires cell-to-cell interaction between CD4+ Th, $CD8^+$ $CD28^-$ T_s and allogeneic APC expressing the class I and class II antigens against which the T cells were primed. Since no suppression occured when mixtures of allogeneic APC were used, it is unlikely that competition between T_h and T_s for the surface of the APC or locally produced IL-2 is the mechanism underlying suppression (18). The role of other lymphokines such as IL-4, IL-10 and TNF-β can also be excluded as antibodies to these lymphokines did not block suppression. Suppression was an early event as it did not occur when the addition of T_s was delayed by more than 8 hours after T_h stimulation.

The need for cell-to-cell interaction between T_h, T_s, and APC, suggests that T_s may act by inhibiting costimulatory

signals delivered by the APCs used for priming. This possibility is strongly supported by our finding that the addition of T_s to the cultures inhibited Th-mediated upregulation of CD80/CD86 expression on stimulating APC. Suppression was first detected after 48 hours of co-culturing Th and Ts consistant with the finding that CD80/CD86 down modulation was seen at 24 hours. Therefore, it appears that T_s induce early changes in target APC, by a yet unknown mechanism, which interfere with the upregulation of B7 molecules required for T_h co-stimulation.

The B7 family has been shown to play a critical role in providing T cell costimulation which is required for the induction of maximal proliferation and cytokine production (15-17). It has been shown that T cells are sensitive to quantitative changes in the molecular interactions that contribute to antigen recognition such as those transmitted through the TCR and CD28 cell surface molecules (19). Allospecific Ts may, therefore, display their effector function by preventing the APC from upregulating the surface density of costimulatory molecules to the threshold required for inducting T_h proliferation. Such a mechanism is consistent with the finding that the capacity of T_h to display proliferative responses in the presence of T_s was restored upon ligation of CD28 by anti-CD28 mAb. Previous studies have shown that blocking the interaction of CD28 with CD80/CD86 either by use of anti-CD28 mAb Fab fragments or CTLA-4-Ig, leads to sustained T cell hyporesponsiveness to the specific alloantigen in MLC (20). Both agents blocked T cell alloreactivity and achieved a similar degree of inhibition on naive and memory T cells. However, T cell responsiveness was not completely abolished suggesting that

other CD28- independent costimulatory pathways contributed to T_h alloactivation. Residual T_h proliferation in the presence of suppressor cells was also observed in our experiments, consistent with this possibility.

The fact that the $CD8^+$ subset of lymphocytes contains a $CD28^+$ population with alloantigen-specific cytotoxic activity and a $CD28^-$ population with suppressor activity has been previously described (21). However, the suppressive effect of $CD8^+CD28^-$ cells as tested in primary MLC was found to be specific for HLA-DR antigens of the allogeneic target rather than for HLA class I antigens.áSince CD8 molecule serves as coreceptor for T cells recognizing MHC-class I molecules, it is difficult to understand how allospecific Ts carrying the $CD8^+CD28^-$ phenotype recognize and react against HLA-DR antigens.

The finding of the present study that allospecific $CD8^+$ $CD28^-$ T_s recognize on target APCs HLA class I antigens, and that they suppress the response of $CD4^+$ T_h to HLA-class II antigens of the same APC, inhibiting upregulation of CD80/CD86 expression, provides a reasonable explanation for the suppressor effect.

Several lines of evidence have demonstrated that molecular mimicry, an extensively discussed phenomenon (22), represents the main mechanism for direct allorecognition (23). Alloreactive T cell clones often display dual recognition ability for a nominal antigen and an alloantigen (24,25). This indicates that allogeneic MHC antigens can mimic immunogenic peptide/self MHC complexes which activate self-restricted T cells. It is, therefore, likely that

allo-MHC class I restricted T_s regulate not only T helper/inducer reactivity against allogeneic HLA-DR antigens, but also T_h reactivity against other antigenic peptides bound to self-MHC class II molecules. In this context the molecular characterization of MHC class I bound peptides which activate T_s may have important implications for the treatment of autoimmune and infectious diseases. While suppression of autoimmunity may be accomplished by priming $CD8^+CD28^-$ T cells with suppressor-inducing peptides, ablation of such a population may be required for treatment of certain infectious diseases. The observation that the proportion of $CD8^+CD28^-$ T cells within the $CD8^+$ subset increases in HIV+ individuals as the disease progresses (26), supports the notion that this population of cells has important immunoregulatory function, and that it may depress T_h reactivity.

Very recently it was shown that chronic activation of both human and murine $CD4^+$ T cells in the presence of IL-10 generates antigen specific T_s which produce high level of IL-10 and inhibit T cell responses to allogeneic monocytes. No direct interaction between T_h, T_s and APC was required in this system. These $CD4^+$ T regulatory cells were shown to inhibit antigen-specific immune responses through the secretion of IL-10 and TGF-β (27). Hence, regulatory T cells operating by different mechanisms exist within the CD4 and CD8 subset.

The present study demonstrates for the first time that allospecific and xenospecific T_s can be generated from any individual by <u>in vitro</u> education and expansion. Preliminary experiments indicate that large numbers of T_s can

be obtained in cultures. Since allospecific and xenospecific $CD8^+CD28^-$ T_s inhibit both the recognition and memory response of $CD4^+$ T cells in primary and secondary MLCs, it is possible that generation of Ts can provide a tool for prevention and suppression of transplant rejection. "Adoptive transfer" of autologous $CD8^+$ $CD28^-$ T_s primed in vitro with donor APC may confer specific immunologic tolerance to human recipients of allogeneic or xenogeneic transplants.

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Second Series of Experiments

The shortage of organ donors is an ever increasing problem in clinical transplantation. Although the use of pig organs may offer a solution, there are still several immunological barriers that should be overcome before xenotransplantation can be envisioned. The first is the hyperacute rejection caused by the binding of naturally occurring antibodies and complement, present in primates, to pig endothelial cells. Recent progress in the generation of transgenic pigs expressing human complement-regulatory molecules on vascular endothelium may solve this critical problem (1-4). However, T helper cell recognition of xenogeneic MHC antigens via the direct and indirect pathways is likely to result in strong cellular immune responses that may be difficult to suppress using currently available strategies (4, 5). It is therefore apparent that the development of methods for specific suppression of xenograft rejection is an important objective for achieving successful xenotransplantation.

Although immunologic tolerance to allogeneic and xenogeneic tissues has been induced in a variety of experimental models (2,6), attempts to ablate specifically the immune response to HLA-incompatible transplants in human patients have failed thus far. However, two recent reports have described strategies for <u>in vitro</u> education of regulatory T cells that suppress in a specific manner the direct recognition by $CD4^+$ T cells of MHC class II antigens expressed on allogeneic APCs (7, 8). In one of these studies, regulatory T cells

with suppressor activity were generated by stimulation of $CD4^+$ T cells with allogeneic monocytes in the presence of IL-10. These regulatory T cells inhibited specifically the reactivity of $CD4^+$ T helper cells through the secretion of IL-10 and TGF-b (7).

In the other study, suppressor T cells were generated by multiple stimulations of human peripheral blood lymphocytes (PBL) with allogeneic APCs and shown to display the $CD8^+CD28^-$ phenotype (8). These $CD8^+CD28^-$ T cells recognized specifically HLA class I antigens expressed by the stimulatory APCs and suppressed the proliferative response of alloreactive $CD4^+$ T cells against APCs used for priming. The suppressive effect was not mediated by lymphokines but instead required cell-to-cell interaction between $CD4^+$ T helper (Th) cells, $CD8^+CD28^-$ T suppressor $(Ts)^3$ cells, and allogeneic APCs expressing antigens against which the T cells were primed. In this system, Ts cells appeared to act by inhibiting costimulatory signals delivered by the allogeneic APCs, such as those provided by CD80/CD86 molecules (8).

This report demonstrates that xenospecific suppressor T cells can be also generated by multiple <u>in vitro</u> stimulations of human T cells with pig PBMCs. The $CD8^+CD28^-$ population from these T cell lines (TCL) recognizes specifically xenogeneic MHC class I antigens and suppresses the proliferative response of Th cells to MHC class II antigens expressed by the xenogeneic APCs. Xenospecific Ts cells interfere with the expression of CD154, the CD40 ligand, on xenoreactive Th cells, further supporting the

concept that the suppressor effect results from inhibition of costimulatory interactions between Th cells and APCs.

The abbreviations used herein are as follows: PI=Propidium Iodide; TCL=T Cell Lines; Ts=T Suppressor cells; SLA = Swine Histocompatibility leukocyte antigen; and CD40L=CD40 Ligand.

Materials and Methods

Pig specimens. Blood was obtained from outbred pigs and from Yucatan miniature swine (Sinclair Research Center, Columbia, MO). MHC haplotypes were defined by RFLP using swine histocompatibility leukocyte antigen (SLA) class I- and class II- specific probes (9-11). For experiments aimed at the identification of MHC antigens recognized by xenospecific Ts cells, blood was obtained from three SLA homozygous lines named W, Z, and Q. Line Q is homozygous for a crossover haplotype that carries the SLA class I genes of strain W and the SLA class II genes of Z (9-11).

Human specimens. Blood was obtained from healthy blood donors typed for HLA class I and class II antigens by conventional serology and by genomic typing of *in vitro* amplified DNA with sequence-specific oligonucleotide probes.

Generation of xenoreactive and alloreactive T cell lines. Human and pig PBMCs were separated from buffy coats by Ficoll-Hypaque centrifugation. Responding human PBMCs ($1x10^6$/ml) were stimulated in 24-well plates with irradiated (1600r) pig or human PBMCs ($1x10^6$/ml). Cells were cocultured for 7 days in complete medium (RPMI 1640 supplemented with

10 % heat-inactivated fetal calf serum (FCS), 2mM glutamine and 50 mg/ml gentamicin) (Gibco, Baltimore, MD). Responding cells were restimulated at seven day intervals in medium containing 10 U/ml rIL-2 (Boehringer Mannheim, Indianapolis, IN).

Cell separation. NK cells were depleted from the alloreactive or xenoreactive TCLs before testing using goat anti-mouse magnetic beads (Dynal, Lake Success, NY) coupled with mAb anti-CD16 and CD56 (Becton Dickinson, San Jose, CA). Suspensions used in blastogenesis assays contained < 2% CD16/CD56-positive cells, as indicated by flow cytometry. $CD4^+$ and $CD8^+$ T cells were separated from alloreactive and xenoreactive TCL by negative selection using Dynal CD4 and CD8 magnetic beads. T cell suspensions used as responders in blastogenesis assays were >98% positive for the CD4 and CD45RO markers. $CD8^+CD28^-$ T cell suspensions were prepared by depletion of $CD28^+$ T cells from purified $CD8^+$ T cell suspensions. For this procedure, goat anti-mouse Dynal beads were coupled with mAb anti-CD28 (Becton Dickinson, San Jose, CA), according to the manufacturer's instructions. The CD28-coupled beads were washed and incubated at $4x10^7$ beads/ml with $1x10^7$ $CD8^+$ T cells for 20 minutes at 4°C, with gentle end-over-end mixing. Rosetted $CD8^+CD28^+$ T cells were detached from the beads by overnight incubation at 37°C and used in cell-mediated lysis experiments. Nonrosetted cells were collected, washed three times and resuspended at $2.5x10^5$ cells/ml in complete RPMI 1640 culture medium. The purity of the suspension was monitored by cytofluorographic analysis. The suspension was rerosetted with CD28 beads when necessary, to obtain a population contaminated by < 2% $CD28^+$ bright cells.

Proliferation Assays. Blastogenesis assays were performed on day 14 or 21, after two or three stimulations, respectively, of human T cells with allogeneic or xenogeneic PBMCs. TCLs were then tested for reactivity to stimulating APCs either as nonfractionated, NK-depleted suspensions ($5x10^4$cells/well) or as NK-depleted $CD4^+$ T cell suspensions ($2.5x10^4$ cells/well). Responding cells were stimulated with irradiated allogeneic or xenogeneic PBMCs ($5x10^4$ cells/well). $CD8^+CD28^-$ T cells tested for suppressor activity were added to the cultures ($1.25x10^4$ cells/well) at the initiation of the blastogenesis assay. To study the dose-dependent effect of $CD8^+CD28^-$ T cells on Th cell proliferation, increasing concentrations of Ts cells were added to parallel cultures as indicated. Cultures were set-up in 96-well trays in a total volume of 0.2 ml. In some experiments, murine mAbs to human IL-10 (at 1μg/ml) or TGF-b (at 5μg/ml) from R&D Systems (Minneapolis, MN) were added to the cultures at the initiation of the assay. After 48 hours of incubation, the cultures were pulsed with $[^3H]$ thymidine ($[^3H]$TdR) and harvested 18 hours later. $[^3H]$TdR incorporation was determined by scintillation spectrometry in an LK Betaplate counter. Results were expressed as mean counts/min of triplicate reactions. Percent suppression was calculated as 1 - [(cpm in Th + Ts + APC cultures)/(cpm in Th + APC cultures)].

Diffusion chamber experiments. Xenoreactive $CD4^+$ T cells (2.5×10^4 cells/well) and irradiated xenogeneic APCs (5×10^4 cells/well) were cocultured in the bottom compartment of a transwell system (Nalge Nunc International, Roskilde, Denmark). Xenospecific $CD8^+CD28^-$ T cells (1.25×10^4

cells/well) were added either to the bottom compartment or cocultured with specific pig APCs in the top compartment of the transwell system. After 48 hours the semipermeable membranes were removed and the proliferative response of Th cells was measured by [^{3}H]TdR incorporation during the last 18 hours of culture.

Flow cytometry. Human T cell subsets were defined using mAb CD4, CD8, CD28, CD45RO, and CD16/56 (Becton Dickinson). Cell suspensions were phenotyped before testing with a FACScan flow cytometer instrument (Becton Dickinson) equipped with a 15-mm argon laser. CaliBRITE flow cytometer beads and FACSComp program (Becton Dickinson) were used for calibration of the cytometer.

To study the expression of CD154 on responding human Th cells, cells were incubated for 6 or 18 hours in MLC and then stained with saturating amounts of mAbs CD3-Per CP (peridinin chlorophyll protein-conjugated anti-CD3 mAb), CD154-PE and CD4-FITC or CD8-FITC (Becton Dickinson).

Cells were analyzed with CellQuest software on a 650 Apple Macintosh computer. Five parameter analysis (forward scatter, side scatter and three fluorescence channels) were used for list mode data analysis. The FL3 channel was used as fluorescence trigger, FL1 and FL2 were used as analysis parameters.

The cytokine profile of xenoreactive Th and Ts cells was determined by flow cytometry. $CD4^+$ Th cells and $CD8^+CD28^-$ Ts cells were isolated from TCLs and activated in 4 hour cultures with 25 ng/ml PMA and 1μg/ml of ionomycin.

Brefeldin A (Sigma Chemical, St. Louis, MO) was added at 10μg/ml for the last 2 hours of incubation to inhibit intracellular transport. Cells were fixed and stained for detection of intracellular cytokines using mAbs IL-2-FITC, IFN-γ FITC, IL-4 PE (Becton Dickinson), and IL-10 PE (R&D Systems).

Study of Apoptosis. The ability of xenoreactive $CD8^+CD28^-$ human Ts cells to induce apoptosis of pig PBMCs and of xenoreactive human $CD4^+$ Th cells after 4 hours of coincubation at 37°C was tested by flow cytometry with the use of annexin V as a marker for apoptotic cells. As positive controls, cells treated with camptothecin (Sigma) were used. The ratio of pig PBMC, human Th cells and human Ts cells was 1:0.5:0.25, as also used in blastogenesis assays. After incubation, cells were stained with mAb anti-human CD3- PE or CD4- PE, washed and subsequently stained with annexin V-FITC and propidium iodide (PI) (R&D Systems). To analyze the population of pig PBMCs, log FL2 (CD3- PE) versus side scatter parameters were used to gate out human $CD3^+$ T cells. The percentage of apoptotic pig cells was determined from log FL1 (annexin-FITC) versus FL3 (PI) dot plots. To analyze the population of human $CD4^+$ Th cells undergoing apoptosis, log FL2 (CD4-PE) versus side scatter parameters were used to gate on CD4 positive cells. Log FL1 (annexin V-FITC) versus FL3 (PI) dot plots of the gated population provided the percentage of apoptotic $CD4^+$ Th cells.

Cytotoxicity Assays. $CD8^+CD28^-$ and $CD8^+CD28^+$ were isolated from activated $CD8^+$ cells and tested for cytotoxicity in a ^{51}Cr release assay. Target cells were pig PBMCs stimulated

with PHA (2μg/ml) 3 days before the cytotoxicity assay. The cytotoxicity assay was performed with different effector to target cell ratios (E:T).

The percent cytotoxicity was calculated as % Lysis =

100 X {[Experimental release (cpm) - Spontaneous release (cpm)]/[Maximum release (cpm) - Spontaneous release (cpm)]}.

TCR Spectratyping

Total RNA was extracted using QIAGEN columns (QIAGEN Inc., Valencia, CA) from xenoreactive human $CD8^+CD28^-$ Ts cells. RNA was reverse transcribed into cDNA in a reaction using Moloney murine leukemia virus reverse transcriptase primed with oligo$(dT)_{18}$ (Clontech Laboratories Inc., Palo Alto, CA), as recommended by the manufacturer.

Aliquots of the cDNA synthesis reaction were amplified in 50-ml reactions with each of the 24 Vβ oligonucleotides (0.5 μM final concentration) and the Cβ oligonucleotide (0.5 μM final concentration). Vβ and Cβ primers were previously described (12, 13). As an internal control for the amount of cDNA used per reaction, a tube containing sense and antisense primers for the first exon of Cβ region was included. Two microliters of the Vβ-Cβ PCR products were subjected to elongation with a fluorophore-labeled Cβ or Jβ-specific primer (0.5 μM final concentration) (12). The size and fluorescence intensity of labeled runoff products were determined on a 377 DNA sequencer (Perkin Elmer Applied

Biosystem Division, Foster City, CA) and analyzed by ABI PRISM 377 GENESCAN Analysis Program (Perkin Elmer Applied Biosystem Division) (13).

The relative intensity of each Vβ family or Jβ-Vβ fragment was calculated as the peak area corresponding to each Vβ family or Jβ-Vβ fragment divided by the sum of all area peaks (12).

Statistical Analysis

Statistical analysis of the results was performed using BMDP statistical software. Analysis of variance to assess significance of group differences (ANOVA) followed by Tukey's method for multiple comparison was applied. Correlation coefficients were obtained using Linear Regression Analysis. Student's *t* test of significance was also used to access the differences between groups.

RESULTS

Specificity of Xenoreactive T Suppressor (Ts) Cells

TCLs were generated by priming T cells from a healthy volunteer (SA) with PBMCs from an unrelated blood donor (BM) or with PBMCs from an outbred pig (pig A). The allospecific TCL (SA-anti-BM) as well as the xenospecific TCL (SA-anti-pig A) showed higher reactivity against APCs from the original stimulator after removal of $CD8^+CD28^-$ Ts cells from the suspensions (Fig. 6). Furthermore, when $CD8^+CD28^-$ T cells were added to the cultures at the initiation of the blastogenesis assay, they inhibited significantly ($p<0.05$)

the reactivity of $CD4^+$ Th cells against APCs used for priming. The suppressive effect was species-specific since $CD8^+CD28^-$ Ts cells primed to pig APCs did not inhibit the response of $CD4^+$ Th cells primed to human APCs. Similarly, Ts cells primed to human APCs did not inhibit the response of CD4 Th cells primed to pig APCs, indicating that Ts cells recognize species-specific antigens (Fig. 6). Studies of an additional four xenospecific and allospecific TCLs yielded similar results.

To determine whether the suppressive effect correlates with the number of Ts cells present in the cultures, Ts cells from two xenoreactive TCLs, MN-anti-pig B and AP-anti-pig B, were tested at various concentrations for their ability to inhibit proliferation of Th cells from TCLs MN-anti-pig B and AP-anti-pig B, respectively. As illustrated in Table 5, the strength of the suppressive effect increased with the number of Ts cells, indicating that suppression was dose dependent ($r = 0.85$, $p < 0.008$).

To determine the nature of the SLA antigens recognized by $CD8^+CD28^-$ T cells on pig stimulating cells, xenoreactive TCLs were generated by stimulating PBMCs from a human blood donor (ES) with irradiated APCs from three different strains of inbred swine: Q, W, and Z. Strain Q shares class I antigens with W and class II antigens with Z, being homozygous for a recombinant haplotype which carries the SLA class I antigens of W and the class II antigens of Z.

Table 6 shows the results of independent experiments in which TCL generated on three different occasions, by priming PBMC from individual ES with APCs from strain Q, W, and Z,

were used.

Table 5.Dose-Dependent Suppression of $CD4^+$ Th Reactivity to Irradiated Pig APCs in the Presence of $CD8^+CD28^-$ Ts.

Number of cells/well			Reactivity (mean cpm) of Th cells from TCL*	
APCs	Th	Ts	MN-anti-pig B	AP-anti-pig B
5×10^4	0	0	278	118
5×10^4	2.5×10^4	0	27,031	13,306
5×10^4	2.5×10^4	1.25×10^4	13,808	6,394
5×10^4	2.5×10^4	2.5×10^4	8,516	2,848
5×10^4	2.5×10^4	5.0×10^4	6,480	2,689

* All reactions were performed in triplicate. The SD to the mean was <10%.

Table 6. Reactivity of CD4+ Th Cells from TCL ES-anti-swine Q in the Presence of CD8+CD28- T Suppressor Cells

Ts from	Source of APCs used for priming of Ts	Genotype of APCs		Reactivity (mean cpm) of CD4+ Th from TCL ES-anti-Q* Source of APCs used in blastogenesis assay			
		Class I	Class II	Q	W	Z	W+Z
Experiment 1							
No Ts added	–	–	–	31,079	3,719	28,269	38,040
ES-anti-Q	Q	W	Z	7,301	1,746	–	32,280
ES-anti-W	W	W	W	12,140	2,711	25,545	–
ES-anti-Z	Z	Z	Z	25,946	2,709	17,018	–
Experiment 2							
No Ts added	–	–	–	33,787	–	32,880	–
ES-anti-Q	Q	W	Z	8,417	–	30,510	–
ES-anti-W	W	W	W	15,240	–	33,150	–
ES-anti-Z	Z	Z	Z	32,790	–	15,208	–
Experiment 3							
No Ts added	-	-	-	22,540	1,452	20,715	19,830
ES-anti-Q	Q	W	Z	5,215	1,310	25,487	20,156
EA-anti-W	W	W	W	7,523	1,640	22,519	9,780
ES-anti-Z	Z	Z	Z	19,834	1,415	8,328	9,530

* For each experiment, reactions were set up in triplicate cultures. The SD to the mean of triplicate reactions was <10%.

In these experiments $CD4^+$ Th cells fromES-anti-swine Q were tested for reactivity in cultures without Ts cells or with Ts cells from ES-anti-Q, ES-anti-W and ES-anti-Z.

The reactivity of Th cells primed to APCs of strain Q to the specific stimulator Q was inhibited efficiently by autologous Ts cells primed to Q or to W (which shares MHC class I antigens with Q), but not by Ts cells primed to Z (which is MHC class II identical, yet class I different from the specific stimulator Q) ($p<0.05$). $CD4^+$ T cell reactivity to strain Z was inhibited only by Ts cells primed to Z, but not by Ts cells primed to strain Q or W which are class I different from Z ($p<0.05$) (Table 6). This indicates that $CD8^+CD28^-$ Ts cells are activated by SLA class I antigens on xenogeneic APCs and inhibit the response of $CD4^+$ Th cells against class II antigens expressed by the same stimulating target cells. The MHC class II specificity of Th cell reactivity was confirmed by the fact that human $CD4^+$ T cells primed to APCs from a strain Q swine reacted to APCs from strain Z (class II identical with Q) but not from strain W (class II different from Q).

To establish whether the suppressive activity of $CD8^+CD28^-$ T cells requires the direct interaction of these cells with the APCs that trigger Th cells reactivity, cell-mixing experiments were performed. In these experiments, mixtures of APCs from strain Z and W were used to stimulate the reactivity of Th cells from TCL ES-anti-Q. The reactivity of Th cells anti-Q was tested in cultures with or without Ts cells primed to Q, W, or Z. In cultures without Ts cells, Th cells primed to Q proliferated vigorously, consistent with the specific recognition of MHC class II antigens

shared by strains Q and Z. This response, however, was not inhibited by Ts cells primed to Q or W, indicating that Ts cells do not inhibit Th cell reactivity to SLA class II antigens unless the SLA class I antigens which they recognize are coexpressed by the same APCs. Indeed inhibition of the response to mixtures of APCs from W and Z was observed only in the presence of Ts cells primed to Z ($p<0.05$), further demonstrating that the interaction of Ts cells and Th cells with the same APCs is required for suppression. This finding is consistent with the hypothesis that Ts cells interfere with the delivery of costimulatory signals by APCs to $CD4^+$ Th cells(8).

It is possible, however, that in addition to interacting with APCs, Ts cells and Th cells also "communicate" with each other, recognizing TCR determinants or other structures in an MHC-restricted manner (14, 15). To explore this possibility, TCLs were generated by stimulating PBMCs from two HLA-disparate individuals, AP (HLA-A30, B35, DRb1*0701, 1301) and MN (HLA-A1, A32, B8, B44, DRb1*0101, 0301) with APCs from the same outbred pig (pig B). The blastogenic response of both TCLs (MN-anti-pig B and AP-anti-pig B) to pig APCs was significantly stronger ($p<0.01$) when $CD8^+CD28^-$ Ts cells were depleted from the cell suspensions, indicating that CD4+ Th cell responses were suppressed by autologous Ts cells (Fig. 7). The reactivity of CD4+ Th cells from both lines to stimulating APCs was inhibited by $CD8^+CD28^-$ Ts cells from either of these lines ($p<0.01$). The difference between the suppressor activity of Ts cells from MN-anti-pig B and AP-anti-pig B was not statistically significant. These results were confirmed in two additional experiments for which other TCL were used. Hence, no MHC-restricted

interaction between Th cells and Ts cells is required for suppression to occur.

To determine whether Ts cells secrete inhibitory factors, coculture experiments using semipermeable membranes, for separating Ts cells from Th cells, were performed. Th cells from TCL ES-anti-W were stimulated with irradiated xenogeneic APC from strain W in the bottom compartment, whereas Ts cells were stimulated with the same APCs in the top compartment. Xenoantigen-specific stimulation of Th cells was inhibited significantly only when Ts cells, Th cells, and APCs were in close contact, but not when Ts cells and Th cells were separated by a membrane (p=0.0001), indicating that cell-to-cell interaction is required for the suppressive effect induced by $CD8^{+}28^{-}$ Ts cells to occur (Fig. 8A).

To further explore the possibility that suppression is mediated by inhibitory cytokines, such as IL-10 or TGF-b, experiments were performed in which mAbs to IL-10 and to TGF-b were added to cultures containing only Th cells, or both Th cells, Ts cells, and stimulating APCs. These mAbs had no significant effect on Th cells proliferation in the absence of Ts cells (data not shown) and failed to abrogate or decrease the inhibitory effect induced by Ts cells on Th cell reactivity (Fig. 8B).

Cytofluorographic analysis of Ts cells from three different TCLs (ES-anti-Q, ES-anti-W and ES-anti-Z) showed that they produced high levels of IFN-γ and moderate amounts of IL-2, yet no detectable levels of IL-4 and IL-10. Th cells from the same cultures produced high levels of IL-2 and IFN-γ,

moderate amounts of IL-4, and no IL-10 (Fig. 9).

Study of Ts Cell-Induced Apoptosis

The possibility that the suppressive activity of xenospecific Ts may be due to killing of pig APCs was explored. Ts cells from a human TCL (GC-anti-swine Z), which inhibited by 88% the response of autologous Th cells to the specific stimulator, were tested for their ability to induce apoptosis or lysis of pig APC. Flow cytometry studies of apoptosis were performed by incubating Ts cells for 4 hours with pig APCs in the presence or absence of xenoreactive $CD4^+$ Th cells and then staining the cultures with annexin V. The percentage of annexin V positive APCs was not significantly different in cultures with or without Ts cells, indicating that no apoptosis of pig APCs was induced (Fig. 10A). Also, the percentage of necrotic pig cells stained by PI was not significantly different in cultures with or without human Ts cells. Furthermore, cell mediated lysis experiments in which PHA-activated pig lymphocytes were used as targets showed lysis when $CD8^+CD28^+$ T cells were used as effectors, but not when $CD8^+CD28^-$ T cells from the same line were tested. This demonstrates that Ts cells do not kill xenogeneic APCs used for priming (Fig. 10B).

Next investigated was the hypothesis that Ts cells may cause apoptotic death of xenoreactive Th cells. In these experiments, $CD4^+$ Th cells from TCL GC-anti-swine Z were incubated for 4 hours with APCs from swine Z, in the presence or absence of autologous Ts cells. The percentage of annexin V-positive $CD4^+$ Th cells in cultures with Ts cells

was not significantly different from the percentage found in cultures without Ts cells, indicating that suppression is not mediated by killing of xenoreactive Th cells (Fig. 10C). Hence, Ts cell suppressive activity is not due to killing of either Th cells or stimulatory APCs.

Expression of CD40 Ligand (CD40L) (CD154) on Xenoreactive Th Cells

The possibility that Ts cells interfere with the costimulatory interaction between CD154 on Th cells (CD40L, T-BAM, p39, or TRAP) and CD40 on xenogeneic APCs has been explored. For this, the expression of CD154 on xenoreactive $CD4^+$ Th cells which were stimulated with pig APCs in the presence or in the absence of Ts cells was studied. After 6 hours of incubation, cells were stained with mAbs anti-CD3, CD154, and either CD4 or CD8. Analysis of the results obtained in independent experiments, using six different TCLs, showed that the level of CD154 expression on $CD4^+$ Th cells was significantly higher ($p<0.01$) in cultures containing pig APCs than in cultures without stimulating cells (Fig. 11A and 11B). However, expression of CD154 on $CD4^+$ Th cells was drastically reduced in the presence of Ts cells (Fig. 11C), indicating that Ts cells prevent antigen-induced up-regulation of CD154 on $CD4^+$ Th cells. There was a statistically significant difference between the level of CD154 expression on Th cell cultures with and without Ts cells ($p<0.01$) in all six experiments. The up-regulation of CD154 was antigen specific, requiring TCR activation, since it did not occur on $CD4^+$ Th cells challenged with APCs from an SLA class II-different pig (Fig. 11D). The expression of CD154 on xenoreactive $CD4^+$ Th

cells was maximal after 6 hours and decreased significantly after 18 hours of incubation with stimulating APCs (data not shown). No expression of CD154 was observed on Ts cells at any time point studied. Hence, Ts cell-induced events that result in Th cell inhibition occur within the first 6 hours of stimulation.

Spectratyping of TCLs Expressed by Ts

The Vβ gene usage of Ts cells from four human anti-pig TCLs (MN-anti-pig B, ES-anti-Q, ES-anti-W, and ES-anti-Z) was determined by spectratyping (Figs. 12 and 13). Ts cells from each of these xenoreactive TCL showed a restricted TCR Vβ gene usage. The side-by-side comparison of the Vb repertoire expressed in unstimulated and stimulated $CD8^+CD28^-$ T cells indicates that after two stimulations with xenogeneic APCs, there was oligoclonal expansion of Ts cells, as illustrated in Fig. 12 and 13.

The Vβ9 and Vβ23 families were expressed by all TCLs yet with different relative intensities (Fig. 12 and 13). The relative intensities of Vβ9 family in TCL MN-anti-pig B, ES-anti-Q, ES-anti-W, ES-anti-Z were 0.22, 0.19, 0.05, and 0.07, respectively, and the relative intensity of Vβ23 was 0.48, 0.38, 0.30, 0.23, respectively. Vβ16 was highly represented in all TCL generated from individual ES yet was absent in TCL obtained from another human subject (MN). Vβ5 was also expressed by Ts cells from all TCL derived from responder ES, but with lower intensity than Vβ16. In two of the TCLs from individual ES (ES-anti-pig W and ES-anti-pig Z), Vβ14 was represented with high intensity (Fig. 13, B and C). Other Vβ families, such as Vβ15 were uniquely

represented in one suppressor cell line (ES-anti-pig Z) (Fig. 13C), while absent from the other lines (ES-anti-Q, ES-anti-W and MN-anti-pig B). Analysis of the CDR3 size distribution revealed a unimodal or bimodal distribution for each Vβ family, except Vβ23 that showed a multipeak gaussian-like distribution (Fig. 14). The oligoclonality of the Vβ repertoire expressed by xenoreactive Ts cells was also confirmed by analyzing the Jβ-Vβ fragments of Vβ families (Fig. 15).

DISCUSSION

The phenotypic characteristics of suppressor cells as well as the mechanisms that underlie their function have been the object of numerous studies (16-25) Both $CD4^+$ and $CD8^+$ T cells with suppressive activity have been described, although antigen-specific suppressor cell lines have been difficult to generate.

$CD4^+$ T cells producing TGF-b, IL-4, and IL-10 were shown to play an important role in protecting animals from experimental autoimmune encephalomyelitis (EAE) after oral feeding with antigen (21). IL-10 was recently shown to induce <u>in vitro</u> differentiation of regulatory $CD4^+$ T cells with suppressor activity and inhibit alloantigen-specific reactivity of $CD8^+$ T cells (7, 23). MHC class-II restricted $CD8^+$ Ts cells that release IL-4 and suppress Th1 cell proliferation were described in human leprosy (24, 25). In the mouse model, $CD8^+$ Ts cells were also described, yet these cells were restricted by nonclassical MHC class I antigens (Qa-1) expressed by B cells and inhibited Th2 responses by production of IFN- g (18). In other studies, suppression

was mediated by Qa-1 restricted $CD8^+$ T cells, which recognize TCR determinants on the membrane of $CD4^+$ Th cells (14, 15, 25). The mechanism of antiidiotypic suppression involved Th cell lysis or induction of Th cell apoptosis via ligation of Fas (15, 19).

An alternative mechanism of suppression seems to reside in inhibition of TCR-mediated cytotoxicity by $CD8^+CD28^-$ and $CD4^+CD28^-$ T cells which express NK inhibitory receptors (26-28). The inhibitory effect of these killing-inhibitory receptors results from mobilization of protein tyrosine phosphatases on the cytoplasmic tail of killing-inhibitory receptor molecules (28).

In a previous study it was shown that human $CD8^+CD28^-$ Ts cells, which inhibit alloreactive $CD4^+$ Th cells, recognize HLA class I antigens on the surface of allogeneic APCs used for priming (8). The suppression was mediated by downregulation of CD80 and CD86 expression on the allogeneic APCs and, thus, by impairment of their ability to deliver the costimulatory signals required for the activation of $CD4^+$ Th cells in response to HLA class II alloantigens.

The present study demonstrates for the first time that the xenospecific response of human $CD4^+$ Th cells to pig MHC class II antigens can be also suppressed by $CD8^+CD28^-$ T cells immunized in vitro against xenogeneic MHC class I antigens. The suppressive effect was not mediated by idiotypic interactions between xenoreactive Ts cells and Th cells, since Th cells primed to APCs from an individual pig were efficiently suppressed not only by autologous but also by allogeneic human Ts cells immunized against the same SLA

class I antigens.

The possibility that suppression of $CD4^+$ Th cells was mediated by lymphokines secreted by $CD8^+CD28^-$ Ts cells is also unlikely, since the suppressive activity required the interaction between Th cells and Ts cells with the same APCs. Thus, Th cell inhibition occurred only when the immunizing SLA class I and class II antigens were coexpressed on the membrane of stimulating APCs, but not when these antigens were expressed by two distinct populations of APCs. Furthermore, diffusion chamber experiments in which Ts and Th cells were separated by semipermeable membranes showed that Th cell reactivity to xenogeneic APCs was not inhibited, indicating that suppression is not mediated by soluble factors.

Cytofluorographic analysis of $CD8^+CD28^-$ Ts cells showed that these cells produce IL-2 and IFN-g, but not IL-4 and IL-10. Moreover, experiments using mAbs against inhibitory cytokines, such as IL-10 and TGF-b, excluded their contribution to the suppressor effect. Hence, neither the production or consumption of lymphokines by Ts cells (22, 24, 29) can explain their inhibitory effect on Th cells in this system.

In the allogeneic system, it was demonstrated that Ts cells interfere with Th cell-induced up-regulation of B7 (CD80, CD86) expression on APC (8). The interaction between CD40 on APC and CD40L (CD154), a transiently expressed $CD4^+$ T cell molecule, is essential for the induction of accessory molecules on APCs, in particular CD80, CD86, and 4-1BB ligand, and for the initiation of antigen-specific T cell

reactivity (30-35). However, blockade of either CD28/B7 or CD40L/CD40 pathways does not inhibit completely T cell mediated alloimmune responses, indicating that, although interrelated, the CD28 and CD40L pathways serve as independent regulators of T cell responses (36).

The possibility that Ts cells interfere with the expression of CD40L (CD154) on activated $CD4^+$ Th cells has been explored. Cytofluorographic analysis showed that up-regulation of CD154 expression on xenoreactive $CD4^+$Th cells was induced by pig APCs, indicating that human CD154/pig CD40 interaction contributes to the strong proliferative response occurring on recognition by human TCRs of SLA class II antigens. Hence, in the human-pig system, xenoantigen-specific $CD4^+$ Th cell responses involve not only the CD28/B7 and CD2/LFA1 costimulatory pathways, as previously described (37), but also the CD154/CD40 pathway. However, the expression of CD154 on xenoreactive $CD4^+$ Th cells was significantly reduced in the presence of Ts cells. The molecular mechanism of CD154 down-regulation on xenoreactive $CD4^+$ Th cells by Ts cells is currently under investigation. The possibility that Ts cells prevent up-regulation of CD40L on CD4+ T cells by killing the xenogeneic stimulating cells or by inducing Th cell apoptosis was ruled out since no evidence of Ts cell-induced cell death was found by either flow cytometry of ^{51}Cr release studies. Proliferation of $CD4^+$ T cells was not restored in the presence of cells expressing constitutively CD40L, suggesting that costimulation of xenogeneic APCs through the CD40-CD40L pathway is not sufficient to circumvent the suppressive effect of Ts cells (A.I. Colovai, manuscript in preparation). Down-modulation of CD154 by Ts cells may

lead, however, to disengagement of Th cells from the targets, preventing full activation and proliferation of these cells.

T cell reactivity to allogeneic and xenogeneic MHC antigens bears resemblance to TCR activation by nominal antigens and pathogens, as it involves recognition of targets expressing novel MHC/peptide complexes. Since the generation of allo-or xenospecific Ts cells in vitro requires multiple rounds of stimulation, it is possible that the chronic exposure to antigen is also required in vivo for the induction of Ts cells. The oligoclonal expansion of a few TCR Vβ families observed within the population of xenoreactive Ts cells is reminiscent of the skewed TCR repertoire displayed by T lymphocytes with HLA class I-specific NK-inhibitory receptors (27), a phenomenon suggested to result from chronic antigenic stimulation.

MHC class I-restricted Ts cells may play a physiologic role in regulating the immune response of Th cells against self or non-self peptide/MHC class II complexes. The finding that there is cross-talk between the MHC class I and class II pathways of peptide processing supports the notion that the same APCs present both helper- and suppressor-inducing peptides (38). It is possible that recognition by Ts cells of MHC class I-bound peptides helps control local inflammation caused by antigen-specific Th cells. Identification of suppressor-inducing peptides may be useful for induction of unresponsiveness to auto-, allo-, or xenoantigens. Furthermore, understanding of the mechanism of Ts cell-mediated down-regulation of CD154 expression on activated Th cells may contribute to the development of new

immunotherapeutic strategies.

This issue becomes particularly important in view of the recent finding that Th cells condition the APCs to directly stimulate T killer cells by CD154-CD40 signaling, rather than by delivering short range acting lymphokines (39-42). The emerging picture from our studies is that Ts cells down-regulate the immune response by interfering with CD154-CD40 signaling, thus preventing the up-regulation of costimulatory (B7) molecules on APCs.

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Third Series of Experiments

INDUCTION OF MHC-CLASS I RESTRICTED HUMAN SUPPRESSOR T CELLS BY PEPTIDE PRIMING IN VITRO

A major goal in the treatment of T-cell mediated autoimmune diseases and allograft rejection is the development of antigen-specific immunosuppression. Induction of antigen-specific T suppressor cells may offer a means of preventing or treating pathogenic responses to self and allogeneic antigens without the complications associated with general immunosuppression.

Although suppressor T cell lines (TCL) are difficult to generate, the existence within the $CD4^+$ and $CD8^+$ T cell population of a functional subset of T lymphocytes that act to downregulate the immune response is well documented (1-5). Different mechanisms have been implicated, yet the cellular and molecular basis of antigen-specific suppression is still unclear (1-5). Recently, it has been demonstrated that allospecific as well as xenospecific Ts can be generated by in vitro priming of human T cells with APCs from an individual of the same (allogeneic) or different (xenogeneic) (6) species (7). Allospecific and xenospecific(6) Ts derive from the $CD8^+CD28^-$ subset and recognize specifically the MHC-class I antigens expressed by the APCs used for priming (7).

Allospecific Ts prevent the upregulation of B7 molecules on target APCs, interfering with the CD28-B7 interaction required for T helper (Th) activation. Furthermore, these Th show no upregulation of the CD154 (CD40L) molecule when stimulated with the priming APCs in the presence of Ts,

indicating that Ts also inhibit the CD154/CD40 costimulatory pathway (6) .

Cell mixture experiments showed that suppression of Th alloreactivity (7) and Th xenoreactivity (6) occurred only when the stimulatory APCs co-expressed MHC-class II antigens, recognized by the Th to be suppressed, and MHC-class I antigens, recognized by the suppressor population. Hence the interaction of the APC with both Ts and Th is a necessary requirement for the development of the suppressor effect.

However, T cell reactivity against both allogeneic and xenogeneic MHC antigens in MLC, occurs primarily via the direct recognition pathway, which is not restricted by the responder's self MHC antigens. Ts-induced alterations of the APCs used for priming may, therefore, represent a pecularity of the direct recognition pathway.

The aim of the present study was to determine whether MHC-class I restricted T cells with suppressor function can be also induced by T cell priming in vitro with nominal antigens such as Tetanus Toxoid and synthetic peptides.

Reported in the third series of experiments is the in vitro generation and characterization of self MHC restricted Ts which recognize antigens that have been processed and presented by autologous APCs.

The following abbreviations are used herein: T helper determinants - HD; Mean fluorescence intensity - MFI; Suppressogenic determinants - SD; Recombinant Tetanus Toxoid

- rTT; tat-DR4 comprising residues 49-57 of HIV-1 tat and residues 64-88 of DRB1*0401 - Tat-DR4 chimeric peptide; T cell lines - TCL; T suppressor lymphocytes - Ts.

Materials and Methods

Peptide Synthesis and Ag Preparations

Recombinant Tetanus Toxoid (rTT) C fragment was obtained from Boehringer Mannheim (Indianapolis, IN) and conjugated to carboxylated polystyrene microparticles (Latex beads; Polysciences, Inc., Warrington, PA) using the Carbodiimide Kit according to the manufacturer's instructions.

A chimeric peptide tat-DR4, comprising residues 49-57 of HIV-1 tat and residues 64-88 of DRB1*0401 was purchased form Chiron Technologies, Australia. The purity of the peptide was >85% as determined by reverse-phase HPLC. The amino acid sequence of this peptide is as follows: RKKRRQRRRQKDLLEQKRAAVDTYCRHNYGVGES.

HLA Typing

Lymphocytes were typed for HLA class I antigens by conventional serology. The class II genotype of the cells was determined by genomic typing of in vitro amplified DNA with sequence-specific oligonucleotide probes for DRB1, DQA1, and DQB1. The HLA phenotype of the blood donor (PR) used in these studies is: HLA-A29, A32, B40, B44, DRB1*1101, DRB1*0701.

Generation of Antigen Specific T Cell Lines (TCL)

PBMCs from a healthy blood male (PR) were separated from buffy coats by Ficoll-Hypaque centrifugation. Responding PBMCs at 2 x 10^6/ml were stimulated in 24-well plates (Nunc, Inc., IL) with 10 μg/ml of tat-DR4 peptide or with 50 μl (approximately 1mg) of rTT-beads in RPMI 1640 medium supplemented with 10% human serum (Sigma Chemical Co., St. Louis, MO), 2mM L-glutamine, and 50 μg/ml gentamicin (Gibco, Grand Island, NY). On day five, 20 U/ml of rIL-2 (Boehringer Mannheim, Indianapolis, IN) were added. Ten days after priming, T cells (2 x 10^6/ml) were collected, washed and restimulated with antigen in medium containing 20 U/ml of rIL-2 and irradiated (3000 rad) autologous PBMCs (2 x 10^6/ml). Antigen specific T cell lines were obtained after two or three restimulations. Two cell lines, named PR-anti-rTT and PR-anti-tat-DR4 peptide were used in this study.

Cell Isolation and Culture

$CD4^+$ and $CD8^+$ T cells were isolated from TCL PR-anti-rTT by positive selection using Dynal CD4 and CD8 beads according to the manufacturer's instructions. After 30 minutes of incubation at 4°C, the rosetted cells were washed and resuspended in 0.1ml of medium containing 15 microliters of DETACHaBead CD4/CD8. After 3 hours of incubation at 37°C, non-rosetted cells were collected, washed, and resuspended in medium.

$CD4^+$ and $CD8^+$ T cells from TCL PR-anti-tat-DR4 peptide were separated by negative selection using Dynal CD4 and CD8

beads. $CD8^+CD28^-$ T cell suspensions were obtained from both TCLs by depleting $CD28^+$ T cells from purified $CD8^+$ T cell suspensions. For this procedure goat-anti-mouse Dynal beads were coupled with mAb anti-CD28 (Becton Dickinson, San Jose, CA) according to the manufacturer's instructions. The CD28-coupled beads were washed and incubated at 4×10^7 beads per ml with 1×10^7 $CD8^+$ T cells for 20 min at 4°C with gentle end-over-end mixing. Non-rosetted cells were collected, washed and resuspended in complete RPMI 1640 medium.

The purity of the $CD4^+$ and $CD8^+CD28^-$ suspensions used in blastogenesis assays was greater than 98% as indicated by cytofluorographic analysis.

Proliferation Assay

Antigen-specific $CD4^+$ T cells or $CD8^+CD28^-$ T cells (30,000/well) obtained from TCLs were cultured with irradiated (3000 rad) autologous PBMCs, as APCs (30,000/well) in 96-well round bottom microplates (Nunc, Inc. Narperville, IL). T cell stimulation with rTT was accomplished using APCs which were pulsed with rTT (5 μg/ml) for 3 hours, then washed and irradiated. tat-DR4 peptide was used at a concentration of 1 μM. $CD8^+CD28^-$ T cells were tested for suppressor activity (30,000 cells/well) by addition to cultures containing Th cells (30,000/well) at the initiation of the blastogenesis assay. The cultures were labeled with $[^3H]$ TdR (0.5 m μCi/well) after 48 hours of incubation and harvested 18 hours later. $[^3H]$ TdR incorporation was then measured in a LK Betaplate liquid scintillation counter (Wallac, Inc., Gaithersburg, MD).

Mean cpm of triplicate cultures and standard deviation of the mean were calculated. Standard deviations were less than 10% of the mean.

Antibody Blocking Assay

Monoclonal antibodies against HLA class I molecules were added to the cultures at the initiation of the proliferation assays. The human mAb OK4F9 (anti-HLA-A29), mAb OK3C8 (anti-HLA A32), mAb 13E12 (anti-HLA-B44) and mAb Ha2C10B12 (anti-HLA-B40) were used as cell culture supernatants. All antibodies were dialysed against RPMI 1640 medium before use.

TCR Spectratyping

Total RNA was extracted using QIAGEN columns (Qiagen Inc. Santa Clara, CA) from $CD8^+CD28^-$ Ts isolated from TCL PR-anti-rTT. RNA was reverse transcribed into cDNA using MMLV reverse transcriptase and primed with oligo $(dT)_{18}$ (Clontech Laboratories Inc., Palo Alto, CA) as recommended by the manufacturer. Aliquots of the cDNA synthesis reaction were amplified individually with 24 human Vb and the Cb primers that have been previously described (8,9). As an internal control for the amount of cDNA used for each Vb PCR reaction, a second reaction tube containing sense and antisense primers for the first exon of Cb region was included. A run-off reaction of the Vb-Cb PCR products was performed using a fluorochrome labeled Cb-specific primer. The size and fluorescence intensity of labeled run off products were then determined on a 377 DNA sequencer (Perkin Elmer Applied Biosystem Division, Foster City, CA) and

analyzed using the ABI PRISM 377 GENESCAN Analysis Program. The relative intensity of each Vb family was calculated as the peak area corresponding to each Vb family divided by the sum of all area peaks.

Flow Cytometry

T cell subsets were defined using mAb CD4-PerCP, CD8-FITC, and CD28-PE from Becton Dickinson, CA. Cell suspensions were phenotyped prior to use in blastogenesis assays using a FACScan flow cytometry instrument (Becton Dickinson, San Jose, CA) equipped with a 15mW Argon Laser. To study the expression of CD80, CD86, and CD40 on CD2 depleted PBMCs, i.e. $CD20^+$ B cells, $CD14^+$ monocytes and dendritic cells, used for antigen presentation, cells were incubated with saturating amounts of mAbs recognizing CD80-PE, CD20-FITC, CD14-FITC, CD86-PE. and CD40-PE (all from Becton Dickinson, San Jose, CA). CD20 postive and CD14 positive cells were gated in and analyzed for CD80, CD86, and CD40 expression. Five parameter analysis (forward scatter, side scatter and three fluorescence channels) were used for list mode data analysis. FL1 channel was used as fluorescence trigger, FL2 as analysis parameter. Mouse IgG (g1 and g2) reagents were used as isotype controls for nonspecific binding of test reagents and as markers for delineating the positive and negative populations. Calibrite flow cytometer beads (Becton Dickinson, San Jose, CA) and FACSComp program were used for calibration of the cytometer.

Results

Inhibition of Th reactivity to Tetanus Toxoid by Ts Cells

In previous studies it was demonstrated that xeno(6) and allospecific Ts recognize MHC-class I antigens on stimulating APCs (7). In order to direct soluble rTT protein to the endogenous antigen processing pathway, which supplies MHC-class I bound peptides, rTT was conjugated to microscopic beads (10).

To determine whether the Th response to a nominal antigen is suppressed by autologous Ts, PBMCs from a healthy individual (PR) were primed in vitro with rTT conjugated beads. $CD4^+$ Th and $CD8^+CD28^-$ Ts, were isolated from the resulting TCL (TCL PR-anti-rTT) and tested for reactivity to rTT in the presence of irradiated autologous PBMCs. $CD4^+$ Th cells reacted vigorously to autologous APCs pulsed with rTT, while $CD8^+CD28^-$ T cells showed no proliferative response (Fig. 16).

When Th and Ts cells from TCL PR-anti-rTT were mixed together at a 1:1 ratio and primed with rTT, there was significantly less Th proliferation than in control cultures in which no Ts were added. The amount of inhibition seen in four repeat experiments ranged from 34 to 37% (Fig. 16). The inhibitory effect of Ts on Th reactivity to rTT was not due to competition for IL-2, as $CD8^+CD28^-$ Ts obtained from the allopeptide specific TCL (TCL PR anti-tat-DR4) caused no inhibition of Th reactivity to rTT (data not shown). Irradiation (3,000 rad) of $CD8^+CD28^-$ Ts cells prior to co-culture with Th and APCs showed no effect on suppressor activity, indicating that the suppressor cells are not radiation sensitive (data not shown).

Inhibition of Th reactivity to Tat-DR4 Peptide

Experimental evidence demonstrates that in monocyte/ macrophage APCs there is communication between the exogenous and endogenous pathways of antigen processing and that antigens in the extracellular millieu can also be presented in association with MHC class I molecules (11). Recently, it was shown that the entry of exogeneous proteins into the MHC class I pathway can be facilitated by conjugating proteins to a short cationic peptide derived from HIV-1 tat (residues 49-57) (12). Based on this knowledge a chimeric peptide consisting of residues 49-57 of tat and residues 64-88 of the DRB1*0401 molecule was synthesized. The latter peptide, which corresponds to the third hypervariable region of the DRB1*0401 antigen, was previously shown to comprise the dominant epitope of the DR 0401 antigen recognized by T cells from individuals carrying the DR*1101 and DR*0701 alleles (13).

To achieve coexpression of Th and Ts epitopes on the same APCs the Tat-DR4 peptide was used for <u>in vitro</u> immunization of T cells from PR (who is DR1101/DR0701 heterozygous).

$CD4^+$ and $CD8^+CD28^-$ T cells were isolated from TCL PR-anti-tat DR4 and tested alone or together for reactivity to peptide tat-DR4 presented by autologous APCs. Blastogenesis assays showed that peptide tat-DR4 stimulated the proliferation of $CD4^+$ T cells but not of $CD8^+CD28^-$ T cells. In the presence of $CD8^+CD28^-$ T cells the response of $CD4^+$ Th was suppressed by >70% in four repeat experiments (Fig. 17). $CD8^+CD28^-$ T cells from this line (PR-anti-tat-DR4) did not inhibit the reactivity to rTT of $CD4^+$ T cells from TCL PR-anti-rTT. Similarly, $CD8^+CD28^-$ Ts cells obtained from TCL PR-anti-rTT

did not inhibit the reactivity of Th from TCL PR-anti-tat-DR4 to tat-DR4, indicating that the suppressor effect is antigen-specific (data not shown).

To determine the MHC-restriction element required for peptide recognition by Ts blocking studies were performed using mAbs specific for the HLA-class I antigens expressed by PR (HLA A29, A32, B40 and B44). The blastogenic response of Th cells to peptide tat-DR4 was of the same order of magnitude in cultures with and without anti-HLA class I mAbs (Fig. 18A). Ts cells inhibited the reactivity of Th cells to the peptide in cultures without mAbs or with mAbs to HLA-A29 or B40. Hence, blocking of HLA-A29 or B40 by mAbs did not prevent Ts activation, indicating that HLA-A29 and B40 molecules did not present suppressogenic determinants of peptide tat-DR4. The suppressor effect, however, was completely abolished when mAbs specific for HLA-A32 or B44 were added to the assay (Fig. 18B) This indicates that the peptide(s) recognized by Ts are presented by HLA-A32 and B44.

Spectratyping of Vb Genes Expressed by $CD8^+CD28^-$ Ts from TCR PR-Anti-TT

Study of the TCR-Vb gene repertoire expressed by xenospecific Ts showed an oligoclonal expansion of a few Vb families.(6) To determine whether the Vb repertoire used by rTT-specific Ts is also restricted the expression of Vb families in the population of $CD8^+CD28^-$ Ts derived from TCL PR-anti-rTT was analyzed. $CD8^+CD28^-$ Ts were sorted from the TCL after two stimulations with rTT, and then expanded, by weekly restimulation with rTT-pulsed APCs, in

IL-2-containing medium. Blastogenesis assays using Th and Ts showed that the $CD8^+CD28^-$ population maintained suppressor activity throughout five consecutive cycles of antigenic stimulation. T cells obtained at later times showed no suppressor activity when co-cultured with $CD4^+$ Th.

Analysis of the TCRs expressed by suppressor cells propagated for five weeks in culture showed that Vb3, Vb5, Vb13, Vb15, and Vb19 were expressed with a relative intensity greater than 10%. The other Vb families were either completely absent (such as Vb6, Vb8, Vb10, Vb11, Vb18, Vb20, and Vb21), or expressed with a relative intensity lower than 5%. After an additional week of expansion, the $CD8^+CD28^-$ population lost suppressor activity and expressed only the Vb3 and Vb5 families (Fig. 19). The loss of Vb13, Vb15 and Vb19 coincided with the loss of suppressor activity suggesting that they represented the Ts population.

Spectratyping of the TCRs used by $CD4^+$ Th cells from TCL PR-anti-rTT showed expression of Vβ3, Vβ5, Vβ9, Vβ10, and Vβ11. Two of the Vβ families (Vβ3 and Vβ5) represented in the $CD4^+$ T cell population, were also found within the $CD8^+CD28^-$ population (where they apparently had no Ts function). Usage of Vβ9, Vβ10, and Vβ11 was unique to rTT-specific Th cells. This finding suggests that the determinants recognized by Th and Ts on the rTT molecule are distinct.

<u>Effect of Antigen Specific Ts on the Expression of CD80, CD86, and CD40 on APCs.</u>

In previous studies it was shown that allospecific Ts downregulate the expression of CD80 and CD86 on the APCs used for priming, interfering with the delivery of costimulatory signals required for Th activation (7). To establish whether such a mechanism is also involved in Ts-induced-downregulation of Th responses to nominal antigens analysed was the expression of CD80, CD86, and CD40 on antigen-pulsed APCs, cultured for 24 hours in the presence, or absence of Ts.

For these experiments $CD4^+$ Th (5×10^5) were mixed with $CD8^+CD28^-$ Ts (1.5×10^6) and APCs (5×10^5) in medium containing tat-DR4 peptide (1 mM). Control cultures containing only APCs or APCs and Ts were set up in parallel. Within 24 hours the expression of CD40 on APCs increased dramatically in the presence of Th as indicated by the shift of the Mean Fluorescense Intensity (MFI) from 633 to 2230, in cultures without, and with Th respectively. However, when Th were co-cultured with Ts, the level of CD40 expression on APCs was about 50% lower (MFI of 1114) than in the absence of Ts (Fig. 20).

Similarily, the level of CD80 and CD86 expression on APCs was greatly enhanced by Th, while Ts induced only a slight elevation of CD80 and CD86 expression. When Ts were added to Th cultures, the expression of CD80 and CD86 remained at the level seen in the absence of Th (Fig. 20). This indicates that Ts interfere with the Th-induced upregulation of costimulatory molecules (CD40, CD80, and CD86) on APCs.

Discussions

Two general approaches to antigen-specific immunotherapy have been proposed (2). The first is to block the activation of T helper cells using MHC blocking peptides, tolerogenic concentrations of antigen or TCR antagonists (2). The second approach involves the induction of antigen-specific regulatory T cells which downregulate immune responses at inflammatory sites (2). Although T suppressor cells have been induced in a variety of experimental models their role and mechanism of action are not well understood. In both mouse and human systems, suppressor cells have been shown to derive from CD4 as well as CD8 subsets (1-5). It has been suggested that $CD4^+$ T cells act as suppressor inducers, while $CD8^+$ T cells act as suppressor effectors (1). A number of cytokines, including interferons, prostaglandins, tumor necrosis factor, transforming growth factors, and interleukin-10 were shown to exert suppressive activity on the growth, differentiation and effector functions of T lymphocytes(14-16).

Suppression generated by oral tolerance to certain autoantigens is antigen and disease specific. This observation suggests that the secretion and action of cytokines must occur in the microenvironment where the immune response is stimulated (2, 14, 15).

Although suppressogenic determinants (SD) of well-defined antigenic proteins have been shown to be different from immunogenic T helper determinants (HD) it has been suggested that they must be localized on a single processed antigenic fragment (1, 17-19) and/or presented by the same APC in a multi-cell cluster for suppression to occur (20, 21).

The concept that the interaction between Ts and Th cells is regional in nature and requires proximity of suppressogenic and immunogenic determinants, is also supported by previous studies from our laboratory showing that in the allogeneic and xenogeneic system, suppression requires an antigen-mediated tripartite interaction between Ts, Th and APCs. It was demonstrated that suppression is specific for MHC-class I/peptide complexes expressed by the APC used for priming and results in diminished T helper cell reactivity to MHC-class II antigens co-expressed by the same APCs (7). This effect was caused by suboptimal costimulation of alloreactive or xenoreactive $CD4^+$ Th in the presence of $CD8^+CD28^-$ Ts. Hence Ts induce downregulation or inhibit upregulation of B7 molecules (CD80 and CD86) on priming APCs and of CD154 (CD40L) on activated Th. Cell-mixture experiments in which Ts and Th cells were co-cultured with two different APCs, one expressing the MHC-class I antigens recognized by Ts, and the other, the MHC-class II antigens recognized by Th, showed no suppression indicating that SD and HD must be presented by the same APC to reveal suppression (6,7).

The present study demonstrates that Th recognition of nominal antigens is subjected to the same mechanism of down-regulation by MHC-class I restricted $CD8^+CD28^-$ cells. Study of the TCR-repertoire used by rTT-specific Th and Ts cells showed overlap of only two TCR Vb families (Vb3 and Vb5), neither of which seemed to contribute to the suppressor activity of the $CD8^+CD28^-$ population. This lack of overlap between the Vb reperoire used by rTT-specific Th and Ts is consistant with the restriction of $CD4^+$ Th cells to MHC class II and $CD8^+$ Ts cells to MHC class I molecules and

with the hypothesis that HD and SD determinants are distinct (1).

Additionally, the oligoclonality of the rTT-specific Th and Ts populations suggests that, in spite of its complexity, the TT molecule has a limited number of immunogenic and suppressogenic determinants recognized by T cells in vitro.

To explore the possibility that exogenous antigens can be used for induction of MHC-class I restricted T suppressor cells a chimeric peptide consisting of residues 49-57 of HIV-tat, which facilitates entry into the endogenous antigen processing pathway, and residues 64-88 of the DR 0401 molecule was used. Processing of the chimeric tat-DR4 peptide by APCs resulted in presentation of both Ts and Th determinants. Ts generated in this system inhibited Th proliferation in an antigen-specific manner. Blocking studies using monoclonal anti-HLA-class I antibodies, demonstrated that the determinant(s) recognized by Ts were presented by two of the responder's HLA class I molecules (HLA-A32 or B40). Since the binding motifs of these two HLA class I molecules differs it is likely that the determinants which $CD8^+CD28^-$ T cells recognize on each of them are not the same. Similarly the amino-acid residues of the tat-DR4 peptide which contact the TCR of Th and Ts cells are probably different due to structural differences between MHC-class I and class II molecules. However, it remains to be studied whether differences in peptide presentation by MHC class I and class II antigens explain the differences in the TCR repertoire and in the selection of suppressogenic and immunogenic determinants.

Cytofluorometric analysis of CD40, CD80, and CD86 molecules on APCs co-cultured with Th and Ts showed that the upregulatory effect exhibited by Th on the expression of costimulatory molecules is drastically inhibited in the presence of Ts. Although the mechanism accounting for the effect of Ts on APCs is still under study, these data suggest that inhibition of Th proliferation is secondary to downregulation of the costimulatory potential of APCs. The finding that Ts downregulate the expression of CD40 on APCs during the tripartite interaction with Th is particularly important in light of the recent demonstration that CD40 is also required for the tripartite interaction between Th, cytotoxic T cell lines (Tc), and APC (22-25).

The finding presented here that Ts cells which inhibit Th reactivity to allo and xenogeneic cells as well as Th reactivity to protein antigens can be educated in vitro has significant implications for the development of antigen specific therapy for treatment of allograft rejection and autoimmune diseases. It is conceivable that autologous Ts generated in vitro can be used for "adoptive" transfer of suppression. Alternatively, active immunization with suppressogenic peptides, targeted to the endogenous pathway may offer a viable strategy for inhibition of indirect allorecognition, a major contributor to rejection, and of autoimmune diseases.

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Fourth Series of Experiments

Inhibition of CD40 Signaling Pathway in Antigen Presenting Cells by T Suppressor Cells

$CD8^+$ T lymphocytes contain precursors of cytotoxic and suppressor cells [1]. The generation of Tc can occur in "sequential two cell interactions"; first, between $CD4^+$ Th cells and APCs and next, between activated APCs and $CD8^+$ Tc cells [2-4]. Another population of $CD8^+$ T cells, which lack the potential of becoming Tc, is characterized by the ability to display antigen specific T suppressor function [1, 5-7]. It has been previously shown that this population of Ts derives from the oligoclonal $CD8^+$ $CD28^-$ subset [8] and recognizes MHC class I/peptide complexes on the cell surface of APCs [5-7].

Allospecific Th and Ts were generated by multiple priming of human T cells with irradiated APCs from MHC class I and class II disparate blood donors. In this system, $CD4^+$ Th, isolated from the T cell line, recognize specifically MHC-class II antigens expressed by the APCs used for priming. $CD8^+$ $CD28^-$ T cells from the same TCL inhibit, in a dose-dependent manner, the proliferative response of $CD4^+$ Th. Inhibition of Th proliferation is not associated with killing of either APCs or $CD4^+$ Th. $CD8^+$ $CD28^-$ T cells recognize specifically MHC class I antigens expressed by the stimulating APCs and render them unable to upregulate the expression of CD80 and CD86 in the presence of Th. This inhibitory effect requires cell-to-cell interactions between Th, Ts and the APCs used for priming [5].

The aim of the present study was to investigate whether the

suppressor effect requires the concomitant interaction between Ts, The and APCs or sequential two cell interactions (first, between Ts and APCs and next, between "suppressed" APCs and The) and whether it is mediated by inhibition of the CD40-signaling pathway.

MATERIALS AND METHODS

Generation and Isolation of allospecific $CD4^+$ and $CD8^+28^-$ T Cell

Peripheral blood mononuclear cells form healthy blood volunteers were primed in MLC with irradiated (1600 rad) PBMCs from MHC-mismatched blood donors. Stimulating cells from the allogeneic donor were depleted of T cells using CD2 magnetic beads (Dynal, New York, NY). After seven days of incubation in complete medium) RPMI 1640 with 10% human serum 2mM L-glutamine and 50μg/ml gentamycin) (Gibco, Grand Island, NY), responding T cells were restimulated with irradiated APCs (CD2-depleated PBMC) from the same blood donor. Three days later $CD4^+$ T cells were isolated by positive selection with Dynal CD4 beads and Detachabeads, according to the manufacturer's instructions. $CD8^+CD28^-$ T cells, from the same culture, were purified first by positive selection of $CD8^+$ T cells with Dynal CD8 beads and Detachabeads, and then by negative selection of $CD28^-$ cells using anti-CD28 mAb coupled to Dynal beads. The separated cells were cultured in complete medium supplemented with 10 μ/ml rIL-2 (Boehringer Manneheim, Indianapolis, IN) for four more days. $CD4^+$ Th cells and $CD8^+CD28^-$ Ts cells were further propagated in IL-2 containing medium by weekly stimulations with irradiated APCs from the same blood donor. Prior to

testing, $CD4^+$ T cells were purified one more time by negative depletion of CD8 cells using CD8 beads. Similarly, $CD8^+CD28^-$ T cells were repurified by depletion of $CD28^+$ T cells using CD28 beads. The purity of $CD4^+$ Th and $CD8^+CD28^-$ Ts was determined by flow cytometry as previously described [5-7].

Proliferation Assays

$CD4^+$ T cells (5 X 10^4/well) from T cell lines were tested for reactivity to irradiated allogeneic APC (2.5 X10^4/well) in the presence or absence of $CD8^+CD28^-$ T cells (2.5 X 10^4/well) from the same TCL. When CD3 antibody was used for T cell activation, the plates were coated overnight with CD3 mAb (1g/ml), then blocked with complete medium and washed. $CD4^+$ and $CD8^+CD28^-$ T cells were used at 5 X 10^4/well. Cultures were pulsed with ^3H thymidine after 48 h of incubation and harvested 18 h later. ^3H thymidine incorporation was measured in an LK Betaplate counter. Mean cpm of the triplicate cultures and SD to the mean were calculated.

Suppression of CD40L Expression on Activated The Cells

Allospecific Th cells were cultured with allogeneic APCs, in the presence or absence of Ts cells for 6 h. mAb CD40L (1g/ml) was added to the culture medium to prevent the rapid internalization of CD40L molecules on the surface of $CD4^+$ T cells [9]. The suspension was washed, stained with FITC-conjugated goat-anti-mouse Ig (Becton Dickinson, Mountain View, CA), then washed and stained with CD4-PE (Becton Dickinson). Four parameter analyses (forward scatter, side scatter and two fluorescence channels) were used for list mode data analysis. Mouse IgG (γ1 and γ2) was used as isotype control for non-specific binding of test reagents

and as markers for delineating the positive and negative populations. CaliBRITE flow cytometry beads (Becton Dickinson) and FACSComp program were used for calibration of the FACScan flow cytometry instrument (Becton Dickinson).

The expression of CD40L on Th activated by use of mAb anti-CD3, in the presence or absence of Ts, was also analyzed after 6 h of incubation by staining the cells with CD40L-PE and CD4-FITC (Becton Dickinson).

Suppression of costimulatory molecules expressed by APC

Crosslinking of CD40 molecules on APCs was accomplished by incubating CD2-depleted PBMCs at 1 X 10^6/ml with an equal number of cells from the $CD40L^+$ D1.1 line [10]. Allospecific $CD8^+$ $CD28^-$ Ts (1 X 10^6/ml) primed *in vitro* to the same APCs were added to parallel cultures. After 24 h of incubation cells were washed, stained and analyzed for expression of costimulatory molecules. The second method for CD40 crosslinking consisted of incubating APCs (2 X 10^6/ml) with FcRII $CD32^+$ L cells (0.5 X 10^6/ml), in medium containing mAb CD40 G28-5 (100ng/ml) and rIL-4 (10 ng/ml) (Boehringer Mannheim). Cultures were set up in parallel with and without allospecific Ts (2 X 10^6/ml). After 48 h of incubation cultures were washed and processed for cytofluorometric analysis.

Aliquots of the same cultures were stained simultaneously with CD20-FITC, CD14-FITC and PE-conjugated mAb specific for one of the following markers: CD40, CD54, CD58, CD80 and CD86 (Pharmingen, San Diego, CA).

To study the kinetics of Ts-mediated suppression of costimulatory molecules induced by the Th, APCs (1 X 10^6/ml) were incubated 48 h with allospecific $CD4^+$ Th cells (2 X 10^6/ml). $CD8^+CD28^-$ Ts (1 X 10^6/ml) from the same TCL were added to the culture at the initiation of the assay or 6 and 18 h later. At the end of the incubation time (48 h), cells were washed and stained with CD20-FITC, CD14-FITC, and CD40, CD54, CD58, CD80, or CD86 PE, as described above.

RESULTS AND DISCUSSION

To define the cellular interactions mediating the suppressor activity of $CD8^+CD28^-$ Ts, the effects of these cells on the earliest events occurring during the program of $CD4^+$ Th activation were studied. An early and critical step in Th activation is the expression of CD40L (CD154)[11, 12]. This molecule interacts with CD40 on APCs and induces APCs to upregulate surface CD80 and CD86 molecules [10, 13, 14].

It is possible that Ts act directly on Th, inhibiting the expression of CD40L or, alternatively, they may act on APCs, blocking the CD40 signaling pathway. To discriminate between these two possibilities, first determined was whether Ts can inhibit Th in the absence of APCs. Experiments in which allospecific Th and Ts were co-cultured in the presence of mAb anti-CD3 showed that Ts do not inhibit Th proliferation or CD40L expression (Figs. 21A, 21B). In contrast, when allospecific Th and Ts are cultured together with the APCs used for priming, both the expression of CD40L and the proliferative capacity of Th are inhibited (Figs. 21C, 21D). These results indicate that the suppressive activity of Ts on Th prliferation is not determined by the direct interaction between Ts and Th and that it requires the

presnece of APCs. This finding is consistent with the previous observation that Ts and Th must recognize the same APC for suppression to occur [5, 6]. It is, therefore, possible that whether APCs can or cannot activate Th depends on their previous encounter with either $CD4^+$Th or $CD8^+CD28^-$Ts.

To explore this possibility, Ts were added to cultures containing allospecific Th and the APCs used for priming, 0, 6 and 18 h after the initiation of the assay. The expression of CD40, CD54, CD58, CD80 and CD86 on APCs was analyzed 48 h, and Th proliferation was measured 72 h, after the initiation of the cultures. In the absence of Ts, Th show strong proliferation in response to stimulation with APCs (Fig. 22) and induce the upregulation of CD54, CD58, CD80 and CD86 on APCs (Fig. 23). In contrast, when Ts were added to the cultures at time 0, Th proliferation was strongly inhibited (Fig. 22) and the level of CD54, CD58, CD80 and CD86 expression on APCs was greatly diminished (Fig. 23). The inhibitory effect of Ts decreased when they were added 6 h after initiation of cultures and was virtually absent when added 18 h later (Fig. 22 and Fig. 23). Taken together these data indicate that suppression is an early event which requires the presence of APCs and that APCs may activate $CD4^+$ Th only if they have not first interacted with Ts.

To determine whether Ts render the APCs unable to stimulate Th, allospecific Ts were incubated with the APCs used for priming. After 6 to 24 h of incubation, Ts were removed from the cultures and the APCs were used for stimulating Th derived from the same TCL. While APCs incubated with naive $CD8^+CD28^-$ T cells elicit strong proliferatin of Th, APCs that

have been incubated with allospecific Ts for 6 to 24 h fail to trigger Th blastogenesis in 3-day assays (Table 7). Propidium iodine and Annexin V staining of the APCs exposed to Ts for 24 h show no loss of viability [5, 6], indicating that Ts do not kill the APCs, yet convert them to a state in which they are no longer capable of sustaining the activation of Th. These data indicate that the costimulatory capacity of APCs is altered by a prior encounter with Ts.

Table 7

Ts render APCs unable to stimulate the proliferation of allospecific Th (^3H thymidine incorporation)

		Medium		Allogeneic APCs preincubated Naive $CD8^+28^-$ T cell		Allospecific CD^+28^- Ts cells	
Cultures	Self-APC	6h	24h	6h	24 h	6h	24h
Th	1.141 ± 67*	40,283 ± 2,941	42,784 ± 3,541	41,965 ± 3,762	44,283 ± 4,023	5,980 ± 548	418±36
Ts	470 ± 45	470 ± 45	532 ± 49	366 ± 41	1,324 ± 142	456 ± 43	64 ± 55
Th +Ts	936 ± 89	7,936 ±689	8,934 ± 815	6,009 ± 524	7,025 ± 673	2,712 ± 231	86 ± 11

*Mean CPM ± SD.

Th or/and Ts from an allospecific TCL were incubated for 3 days with autologous or allogeneic APCs ($CD2^+$ depleted PBMC). The allogeneic APCs used for stimulation were preincubated for 6 or 24 h with allospecific $CD8^+28^-$ cells. The U cells were then depleted from the mixutre with CD2 magnetic beads. The cultures were labled and harvested after 72 h. Results are representative of three different experiments.

Since ligation of CD40 molecules on APCs has been shown to upregulate the expression of costimulatory molecules [10, 13], it is possible that Ts can inhibit the CD40 signaling pathway. To explore this hypothesis two well established systems were used. In one of these systems, upregulation of costimulatory molecules on APCs through CD40 signaling is accomplished by ligation of CD40 molecules on APCs using the D1.1 Jurkat T cell line, that constitutively expresses CD40L [10]. In the other system, CD40 antibody bound to the Fc (CD32) receptor of L cells were added to APCs in cultures containing IL-4 [13]. Crosslinking of CD40 molecules on APCs either by incubation for 24 h with the CD40L positive D1.1 line or by incubation for 48 h with anti-CD40 antibody results in upregulation of CD54, CD58, CD80 and CD86 molecules on APCs (Table 8). CD40 triggering also increases the mean fluorescence intensity (MFI) of CD40 on APCs (Table 8). In contrast, in the presence of allospecific Ts, APCs cultured for the same periods of time with D1.1 T cell line or CD40 antibody failed to upregulate any of these molecules (CD40, CD54, CD58, CD80 and CD86). The data indicate that Ts inhibit the costimulatory capacity of APCs, by interfering with CD40 dependent signaling.

Table 8

Ts inhibit CD40-induced upregulation of costimulatory molecules on APCs

Cultures	%Positive APCs (MFI*)				
	CD40	CD54	CD58	CD80	CD86
APC	99(203)	60(350)	42(374)	17(90)	35(318)
APC + D1.1	99(312)	85(358)	70(380)	41(141)	49(327)
APC + D1.1 + Ts	99(172)	11(147)	14(209)	7(78)	16(178)
APC	99(231)	65(283)	40(315)	16(78)	30(310)
APC + CD 40Ab +IL-4	99(430)	97(443)	80(458)	31(109)	63(464)
APC + CD 40Ab +IL-4 + Ts	93(201)	56(192)	22(263)	10(47)	33(221)

* Mean channel fluorescence intensity.
APCs ($CD2^+$ cell-depleted PBMC) were cultured with either $CD40L^+$ D1.1 cells or mAb CD40 bound to Fey receptor of L cells plus IL-4 Ts were added at the initiation of the assay. Expression of costimulatory molecules on $CD14^+$ and $CD\ 20^+$. APC was analysed by flow cytometry. Results are representative of three independent experiments.

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Taken together these data indicate that Ts interacts directly with APCs, inhibitingh CD40-mediated CD80 and CD86 upregulation. The "suppressed" APCs are rendered unable to induce and sustain the full program of Th activation. Thus, Th exposed to "suppressed" APCs fail to upregulate completely CD40L expression due to an aborted "crosstalk", that is normally based on CD40-mediated upregulation of CD80 and CD86 [15, 16].

There is ample evidence that in the absence of co-stimulation TCR interaction with MHC/antigen complexes can lead to T cell anergy [17-19]. T cell anergy can be restored by crosslinking the CD28 molecure or by the use of exogenous IL-2 [17-19]. It was found that addition of rIL-2 to cultures containing Th, Ts and APC restores Th proliferation (Fig. 24). This indicates that Th are rendered anergic by Ts-treated APCs consistent with the previous finding that CD28 crosslinking restores Th reactivity in cultures with Ts [5].

The data herein support a model in which T-cell mediated suppression can result form the sequential interaction between first, TS and APCs and next, "suppressed" APCs and Th

(Fig. 25). In this regard the present findings confirm and extend the "temporal bridging" model recently described to account for the complex role that APCs play in Th-mediated generation of $CD8^+$ Tc[2-4]. Furthermore, the present data complement the finding that CD40 signaling is essential for conditioning APCs, by demonstrating that Ts inhibit this pathway. New data show that Ts inhibit The-induced activation of NF-B in APC, thus interfering with the upregulation of B7 costimulatory molecules (Li, J., Liu, Z., Jiang, S., Cortesini, R., Lederman, S., Suciu-Foca, N. submitted).

Further dissection of the molecular interaction between Ts and APCs should allow the development of new strategies for specific suppression of the immune response in transplantation and autoimmune diseases.

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Fifth Series of Experiments

In the first through fourth series of experiments, we identified and characterized human antigen specific T suppressor cells (Ts). It was shown that Ts inhibits the costimulatory activity of APC blocking NF-κB activation and transcription of costimulatory molecules. To explore the underlying mechanism we used for allostimulating peripheral blood B cells or cells from the dendritic cell line KG-1. Total RNA prepared from KG-1 or from B cells that have been exposed to allospecific Th, Ts of Th/Tz mixtures for 12 hours was used in a cDNA micro-array system to identify genes which are differentially expressed in APC. Although transcription of a wide array of genes was suppressed, expression of 10-15 genes was up-regulated >2-3 fold in APC cocultured for 12 hours with Ts or Ts/Th mixtures. Included in this latter group are the Monocyte Inhibitory Receptor (MIR-10 or ILT4), ILT2 (MIR7), and ILT3. MIR-10, MIR7 (ILT2) and ILT3 belong to a family of leukocyte inhibitory receptors (LIRs) which bear homology to killer inhibitory receptors (KIRs). These molecules interact with MHC-class I molecules via Ig-like domains and regulate negatively the activation of APC, recruiting an inhibitory signaling molecule, thyrosine phosphatase SHP-1. These data indicate that Ts-induced suppression of APC is based on an active mechanism by up-regulating the expression of a class of inhibitory receptors which transmit negative inhibitory signals in APC. Ts provides an essential regulatory mechanism through which immune tolerance can be achieved.

The fifth series of experiments studies the function of MIR-genes which mediate the T effect on APC. Upregulation of

MIR expression renders the APC tolerogenic as they induce Th anergy, rather than Th stimulation. Hence, overexpression of MIRs in APC provides a therapeutic tool for induction of tolerance.

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Sixth Series of Experiments

Using a retroviral vector (MIG) previously used for experimental gene therapy studies, we generated an antigen presenting cell line that can over-express ILT3 and ILT4 proteins in a cell culture system. We have found that antigen presenting cell (APC) over-expressing the ILT3 gene render T helper (Th) cells anergic and drastically lose antigen presenting activities.

ILT3 (1) and ILT4 (2) the amino acid sequences of which and the nucleic acid sequences encoding these proteins are set forth in Figures 33A-33B and 34, respectively,belong to a family of leukocyte inhibitory receptor (LIRs) which bear homology to killer inhibitory receptors (KIRs). These molecules interact with either MHC-Class I like proteins or other unknown ligands and recruit an inhibitory signaling protein, ShP-1.

The subject invention provides methods of producing genetically engineered APCs using the aforementioned vector or any other vector suitable for introducing nucleic acid molecules into cells, and APCs genetically engineered thereby. Such genetic engineering of APCs from transplant donors according to the subject invention renders the APCs capable of inducing tolerance to a graft in transplant recipients (xenograft or allograft) and are therefore, useful in treatment of rejection in transplant recipients. Another important method provided by the subject invention is detecting or measuring the level of expression of ILT3 in a sample from a subject as an index to monitor the appearance of T suppressor (Ts) cells. For example T cells

displaying CD8+CD28- phenotype, which can induce up-regulation of ILT3 in APC, are considered suppressor T cells. Accordingly, one of skill could use such a method of measuring the level of expression of ILT3 to detect the induction of suppressor T cells in transplant recipients.

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Seventh Series of Experiments

T Suppressor Cells Inhibit T helper Induced NF-kB Activation in APC

The existence of immunoregulatory T cells which down regulate, in an antigen-specific manner, the immune response has been demonstrated in a variety of experimental models of transplantation and autoimmune diseases. However the cellular identity of these cells as well as their mechanism of action have remained elusive. It has been postulated that antigen specific suppression may be caused by a) immune deviation through a shift from Th1 to Th2 type cytokines i.e. a shift from pro-inflammatory to anti-inflammatory cytokines, b) generation of IL-10 and TGFβ producing regulatory T cells; c) induction of anergic T cells which compete with immunocompetent T cells for access to the surface of the APC and for locally produced IL-2 or d) generation of anti-idiotypic T cells which kill antigen-specific T cells, recognizing on their membrane certain TCR-derived peptides. [1-7]

In rodents the adoptive transfer of transplantation tolerance from an immuno-suppressed host to a naive recipient has been accomplished experimentally by use of $CD4^+$ T cells. In contrast $CD8^+$ T cells from tolerant animals were found to be unable to transfer donor-specific unresponsiveness.

We have demonstrated that antigen-specific human T suppressor cells have distinct phenotype. They are $CD8^+$ $CD28^-$, MHC - class I restricted and oligoclonal. Alloxeno-

and nominal antigen specific Ts inhibit the proliferation of $CD4^+$ Th with cognate antigen specificity and render bridging APC tolerogenic by a contact-dependent mechanism. [1-7]

First generated were human Ts by multiple stimulation of human PBMC with irradiated APC of allogenic (human) or xenogenic (pig) origin. The resulting T cell live (TCL) showed a progressive decrease in blastogenic responses when challenged in 3-day proliferation assays with APC from the specific stimulator. However, when $CD8^+$ $CD28^+$ T cells were depleted from these cultures, the ability of $CD4^-$ Th to display vigorous proliferative responses to the APC used for priming was restored. Addition of $CD8^+$ $CD28^-$ T cells back to the cultures inhibited the response in a dose-dependent manner. Stimulation of mixtures of allospecific $CD4^+$ Th and $CD8^+$ $CD28^-$ Ts with APC sharing with the original priming cells both class I and class II MHC antigens resulted in dominant suppression, i.e., no Th proliferation occurred. However, Th proliferated vigorously when these mixtures were stimulated with APC which shared only class II antigens with the priming APC. The fact that no suppression occurred in these cultures indicates that while Th recognize MHC class II/peptide complexes, Ts are triggered by MHC class I/peptide complexes. In cultures in which we used APC from two different blood donors, one sharing class I and the other class II with the original stimulator, no suppression of Th proliferation was seen. This demonstrates that suppression requires cell-to-cell interaction between $CD4^+$ Th, $CD8^+$ Ts and APC expressing the class I and class II antigens against which the TCL was primed. Since cell-to-cell contact is required it becomes obvious that the Ts effect is mediated through APC rather than through Th-Ts

interaction and that no cytokines are involved. [1-7]

Using both allospecific and xenospecific TCL it was shown that stimulation with the corresponding APC induces the expression of CD40L on Th. However, CD40L expression on Th was drastically reduced in the presence of Ts. The ability of Ts to suppress the APC-induced upregulation of CD40L was abrogated when with specific anti MHC-class I mAb were added to the culture, further documenting that Ts, recognize MHC class I antigens. [1-7]

It was further demonstrated that Th incubated with Ts for 12 to 24 hours were rendered anergic, as they produced no IL-2 and failed to proliferate when challenged with the specific stimulators. Addition of exogenous IL-2 or anti-CD28 mAb, to cultures containing Th, Ts and APC, restored Th reactivity to priming APC, consistent with the notion that Th were rendered anergic in the presence of Ts.

To determine whether Ts inhibit the costimulatory activity of APC, explored was the expression of CD40, CD80 and CD86 on APC after 48 hours of incubation with Th and/or Ts. Th failed to induce the upregulation of costimulatory molecules on the membrane of APC in the presence of Ts. [1-7]

To characterize the effect of Th and Ts on the expression of steady-state CD86 mRNA levels, APC that had been cultured for 48 hours with Th, Ts, or mixtures of Th and Ts were purified and total RNA was prepared. Densitometry analysis of Northern blots for CD86 mRNA showed that Ts inhibited the Th-induced upregulation of APC steady-state CD86 mRNA levels in a dose-dependent manner. To determine whether the Ts

effect on CD86 expression occurs at the level of transcription nuclear run-on assays were performed. Ts inhibited the Th-induced up-regulation of APC CD86 expression at the level of transcription.

To further characterize the mechanism by which CD86 transcription in APC is regulated by Th and Ts the CD86 promoter was cloned. Two canonical NF-κB binding sites, one at -612 and another at -238 were found. A series of luciferase reporter gene plasmids were generated that represented 5` deletions of the CD86 promoter or which contained a mutated NF-κB consensus binding site at -238 or at -612. The resulting constructs were transfected into APC to measure their transcriptional activities. When exposed to Th, APC transfected with reporters driven by the -1179 and -781-bp DNA elements showed significant increases in luciferase activity. Ts inhibited the Th-induced luciferase activity. Truncation of the -612 NF-κB binding site or mutations demonstrated that CD86 transcription induced by Th in APC depends on the -612 NF-κB consensus site.

Hence, the present study indicates that cell-to-cell interactions between Th and APC induces APC to activate NF-κB which drives transcription of CD80 and CD86. Ts modulate Th reactivity by regulation NF-κB activation in APC.

To further explore the pattern of genes expressed in resting APC and in APC exposed for 12 hours to Ts an experimental model was developed which permitted us to obtain a large number of a relatively pure population of dendritic cells. Human PBMC were primed in vitro with cells from an immature dendritic cell line. After multiple stimulations, TCL which

contained $CD4^+$ Th and $CD8^+$ $CD28^-$ Ts were obtained. Proliferation assays showed that Ts from these TCL inhibited Th proliferation by >80% at a 1:1 Th/Ts ratio. APC incubated for 12 hours with Ts induced Th anergy rather than Th proliferation when used for priming naive or in vitro alloimmunized Th. RNA was isolated from KG1 cells grown for 12 hours with or without Ts. Poly $(a)^+$ mRNA was purified using oligotex mRNA mini kits. cDNA was synthesized from the poly $(A)^+$ mRNA. cRNA was prepared in an in vitro transcription reaction by using T7 polymerase. RNA transcript levels for different genes were assessed using Affymetrix software. More than 100 genes showed a two fold increase or decrease in the level of expression when KG1 cells were incubated for 12 hours with Ts. It was found that the interaction between APC and allogeneic T suppressor cells seems to promote cell survival, since APC showed upregulated antiapoptotic molecules, such as several members of the Bcl family, and the cyclooxygenase 2. Synergically, the expression of proapoptotic molecules decreases, such as ICII-1L also known as caspase 2, the p53 stabilizing kinase Chk2 and the death associated protein kinase DAP-K. Interaction between APC and allogeneic T suppressor cells also induces changes in the expression levels of costimulatory molecules such as 4-1BB/CD137, RANK and RANKL. Other costimulatory molecules, known to play an important role in initiation and maintenance of the antigen specific T cell/dendritic cell interaction were downregulated. T suppressor cell mediated downregulation CD86 has already been described, but along with it, one can now observe downregulation LFA-1 related surface molecules such as of one of its ligands, ICAM-2, one of its interacting molecules, DNAM-1, and P150, 95. Similarly, adhesion

molecules such as integrin aplha6, contactin and N-cadherin were downregulated in APC upon contact with suppressor T cells.

A large number of transcription factor and transcription related molecules were also affected. Of particular interest, transcription factors such as STAT5b, involved in IL-2 signaling, the PI-3K related FREAC-4 and numerous zinc finger proteins were downregulated, whereas proto-oncogenes were upregulated. It had already been shown that NF-κB translocation was inhibited by T suppressor cells. GeneChip data now seem to point to the fact that, although NF-κB expression did not decrease expression of the NF-κB inhibitors I-κB, MAD-3 and A20 was increased upon interaction with T suppressor cells. It is of interest to note that the promoters of CD86 and LFA-1 related molecules which were downregulated after APC interaction with T suppressor cells, contain NF-κB binding sites. At last, although the expression of activation markers such as CD69 was upregulated, key molecules in activation pathways were downregulated such as: dihydroorotate dehydrogenase (DHODH), target of the immunosuppressive drug Lefunomide, Calcineurin B, target of the immunosuppressive drugs FK506 and CsA, PKC delta and PI-3K (phophatidl Inositol 3 kinase), involved in numerous pathways, including that of NF-κB. Of interest, as well, is the downregulation of the two activating members of the LIR/MJR/ILT "inhibitory" receptor family, LIR6 and LIR7.

Along the same lines, detected was an increase in the expression of ITIM motif-bearing inhibitory molecules such as IL-3R, IL-4R, PKC zeta and FcgammaRIIB. Among those, FcgammaRIIB has been best characterized, mainly in the B

cell system, as a powerful inhibitor of costimulation, through a pathway that my involve inhibition of PI-3-K.

Hence, it appears that after the anergizing interaction with T suppressor cells, APC modify the expression of molecules in a way that promotes cell survival and cell cycle. Some indicators of activation can be detected, but others are downregulated, such as CD86 and the LFA-1 pathway, both NF-κB dependent, or calcineurin and PI 30K. However, interaction with T suppressor cells does not only cause downregulation of activating molecules which could be viewed as a "passive anergy". Rather, some inhibitory molecules are upregulated, which are known in inhibiting immune responses. This seems to point in the direction of an "active anergy" of KG-1 cells caused by T suppressor cells.

Having learned that allospecific T suppressors cells inhibit Th mediated upregulation of APC costimulatory molecules, this notion was capitalized on and developed was a method for assessing the presence of Ts in the circulation of transplant recipients. The method consists of quantitating the level of CD80, CD86 and CD54 expression on APC from the transplant donor after 24 hours of incubation with recipient $CD4^+$Th or with mixtures of $CD4^+$ Th and $CD8^+$ $CD28^-$ Ts. Inhibition of membrane expression of costimulatory molecules on APC is considered indicative of suppression. Serial evaluation of 64 liver, 14 kidney and 103 transplant recipients in two different centers (University of Rome "La Sapienza" and Columbia University, New York) showed a highly significant correlation between the presence of Ts and quiescence. The inhibitory effect of Ts showed an exquisite specificity for the HLA class I antigens of the donor.

Taken together the present study demonstrates for the first time the identity and mechanism of action of T suppressor cells. It is shown that MHC-class I restricted Ts develop following alloimmunization and that these cells inhibit upregulation of costimulatory molecules while augmenting the expression of inhibitory receptors, which bear the ITIM motive in APC. These tolerogenic APC render Th anergic. In turn, anergic Th can spread tolerance by suppressing other naive APC with which they interact.

The discovery of the identity of Ts and of their mechanism of action opens new avenues to the development of immunosuppressive strategies.

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Eight Series of Experiments

CD8+CD28- T suppressor cells represent a distinct subset in a heterogeneous population

The population of CD8+CD28- T cells was shown to be increased in aged individuals and patients with AIDS (1, 2). This population contains cytotoxic T cells and T cells with antigen non-specific suppressor function (3, 4). Studies of allospecific and xenospecific human T cell lines (TCL) have revealed the existence of an additional subset of CD8+CD28- T cells which act as antigen-specific, MHC class I-restricted suppressor cells (Ts) (5-7). Ts can be generated by multiple <u>in vitro</u> stimulation of human T cells with allo- or xenogeneic APC and suppress the reactivity of T helper (Th) cells by inhibiting the costimulatory function of antigen presenting cells (APC). In this model, APC are rendered unable to up-regulate the expression of costimulatory molecules CD80, CD86 and CD40 and, thus, to initiate and sustain Th activation. Ts do not induce APC apoptosis and require cell-to-cell contact in order to exert their function (5, 6).

To further characterize antigen-specific Ts, investigated here was the cell cycle and the phenotypic characteristics of CD8+CD28- T cells from allo- and xenogeneic TCL. An experimental system was used in which individual T cells are tagged with a fluorescent dye that segregates equally between daughter cells upon division, allowing the proliferative history of any cell to be monitored over time (8). Using multicolor flow cytometry analysis, this system

permits simultaneous evaluation of cell division and expression of cell surface markers, such as CD8 and CD28.

Material and Methods

Cell preparation and cultures. Human peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density centrifugation from heparinized blood obtained from healthy volunteers. Human allo- and xenoreactive TCL were generated by stimulation of PBMC, plated at 10^6 cells/ml, with irradiated allogeneic or xenogeneic (swine) PBMC ($1x10^6$ cells/ml) in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, and 50 μg/ml gentamycin (Gibco, Grand Island, NY). Responding T cells were restimulated at 7-day intervals in the complete medium with recombinant IL-2 added at 10 U/ml (Boehringer Mannheim, Indianapolis, IN).

Cell cycle studies. Freshly isolated PBMC were resuspended in prewarmed (37°C) PBS containing the vital dye carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) at 500 nM. Cells were incubated for 15 min at 37°C, washed with medium and incubated for another 30 min to ensure complete reaction with intracellular amines. After an additional washing, CFSE-labeled PBMC were incubated with allogeneic CD2-depleted PBMC for 0, 5 or 7 days, and then stained with mAb CD28 PE and CD8 PerCP (Becton Dickinson, San Hose, CA) for flow cytometry analysis. Responding T cells isolated from alloreactive TCL were first depleted of CD4+ cells using CD4 Dynal beads, labeled with CFSE and then incubated with allogeneic CD2-depleted APC from the specific stimulator in the absence or presence of IL-2 (10 U/ml). Aliquots of these

cultures were stained at time 0, 48 and 72 hr with mAb CD28 PE, and analyzed by flow cyotmetry.

Flow cytometry. Multi-color cell analysis was performed on a Becton Dickinson FACScan instrument. Cells were gated based on the expression of the CD8 marker (FL3), and CFSE- and CD28-positive cells were visualized in FL1 and FL2, respectively. For immunophenotypic analysis of antigen-specific suppressors, CD8+CD28- cells were isolated from allogeneic or xenogeneic TCL using Dynal magnetic beads and then stained with the indicated monoclonal antibodies (mAb) (Becton Dickinson, San Hose, CA).

Results and Discussion

To determine whether CD8+CD28- T cells from freshly isolated PBMC are able to proliferate in response to allogeneic APC, PBMC from donor BB1 were labeled with the vital dye CFSE and stimulated in MLC with CD2-depleted PBMC from donor BB2. Aliquots of this culture were stained on day 0, 5, and 7 with mAb CD28 PE and CD8 PerCP, and analyzed by flow cytometry. CD28 expression and simultaneous loss of the vital dye CFSE were measured within the CD8 gated (G1) population. As indicated in Fig. 35, all CD8+ cells were positive for CFSE on day 0. However, on day 5, 16.6% of the CD8+ T cells proliferated in response to allogeneic stimulation, showing loss of CFSE (% upper left quadrant + % lower left quadrant). Among the proliferating cells, CD8+CD28- cells represented only 2.9% of the total CD8+ cells (lower left quadrant), while CD8+CD28+ T cells accounted for approximately 13.7% of the CD8+ cells (upper left quadrant). By day 7, the percent of total CD8+ cells

which had divided increased to 42.7%. Among these cells, CD8+CD28- cells represented 4.4% of the total CD8+ cells (lower left quadrant), while CD8+CD28+ T cells accounted for 38.3% (upper left quadrant). Interestingly, 57% of the CD8+ cells found alive in the culture on day 7 did not proliferate and did not undergo any morphological changes indicative of apoptosis. Also, they did not acquire annexin V expression (data not shown). The failure of a significant proportion of CD8+ T cells to be recruited in the dividing pool may reflect distinct activation requirements of different T cell populations (e.g. different TCR or naive versus memory T cells).

In the next series of experiments studied was the proliferative capacity of stimulated (memory) CD8+CD28- T cells isolated from allogeneic TCLs. TCL BB1-anti-BB2 was generated by two rounds of stimulation of PBMC from individual BB1 with irradiated PBMC from donor BB2. T cells from TCL BB1-anti-BB2 were collected on day 14, subjected to depletion of CD4+ cells, and labeled with CFSE. Cells were then challenged with CD2-depleted APC from donor BB2 in the presence or absence of IL-2, and stained with mAb CD28. Flow cytometry analysis indicated that CD8+ cells did not proliferate after 48 or 72 hr of culture in the presence of APC alone, but proliferated when challenged with APC and IL-2 (Fig. 36). Proliferating CD8+CD28+ represented 19.0 % and 31.0% of the total CD8+ T cells at 48 and 72 hours, respectively. Proliferating CD8+CD28- cells accounted for 9.2% and 52.8% of all CD8+ T cells at 48 and 72 hr, respectively. These results indicate that in contrast to the previously described senescent CD8+CD28- T cells, a significant fraction of the CD8+CD28- subset does have

proliferative capacity. Our data is reminiscent of the finding that human CD8+CD28- T suppressor cells primed in vitro by multiple stimulation with allo- or xenogeneic APC display an oligoclonal TCR VB gene expression (6), indicating preferential expansion of a skewed T cell repertoire. The results here also suggest that CD8+CD28- Ts, that downregulate the immune response, have at first a low proliferating rate, but reach a sizable population upon repeated stimulation with antigen.

The finding that CD8+ T cells which had been repeatedly exposed to antigen fail to proliferate upon antigenic rechallenge unless helper factors, such as IL-2, are present in sufficient amounts is in accordance with previous studies showing that CD8+ T cells become nonresponsive following antigenic stimulation, even in the presence of costimulation (9). Antigen-induced non-responsiveness of CD8+ T cells may be of particular importance in regulating the immune response. When the initial helper-independent CD8+ T cell response is not sufficient to clear the antigen, persistent activation of T helper cells and secretion of helper cytokines will induce the expansion of a population of CD8+CD28- suppressor cells which inihibit the APC function, and thus, control further activation and expansion of Th.

Also studied was the phenotypic characteristics of the CD8+CD28- Ts isolated from alloreactive and xenoreactive TCL, and it was found that their phenotype is as follows: CD3+CD8+CD28-CD45RO+CD45RA-CD25-CD40L-CD18+CD54+BY55-. As opposed to CD8+CD28- cytotoxic T cells, suppressor T cells do not express the BY55 marker (3). Also, in contrast to antigen non-specific CD8+CD28- T suppressor cells, which

recognize the class Ib molecule CD1 on intestinal epithelial cells, antigen-specific Ts recognize MHC class I/peptide complexes expressed by APC (4-7). These findings point to the existence of a distinct CD8+CD28- subset that display proliferative capacity and antigen-specific suppressor function. The ability of these cells to proliferate in vitro may be crucial for the development of new strategies to induce allo- and xenogeneic transplant tolerance.

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Ninth Series of Experiments

Ninth Series of Experiments

CD8$^+$CD28$^-$ T cells Suppress Alloresponse of CD4$^+$ T cells Both in Primates and Rodents

It has been previously shown that allogeneic, xenogeneic and nominal-antigen specific T suppressor cells (Ts) can be generated in vitro by multiple priming of human T cells with allogeneic, xenogeneic and autologous antigen presenting cells (APC) pulsed with antigen (1-3). Ts are contained in a CD8$^+$CD28$^-$ population, recognize MHC-class I/peptide complex on APC and use limited TCR Vβ gene families. Suppression is not caused by direct interaction between Ts and CD4$^+$ T helper cells (Th) or the cytokines secreted by Ts. Ts directly suppress APC, downregulating B7 molecules on APC. Th are then anergized without costimulation when they contact "suppressed" APC. Blocking B7/CD28 interaction has been shown to prevent graft rejection in vivo (4). Ts mediated downregulation of B7 molecules on APC may provide another promising therapy for transplant patients. It is therefore important to have an animal model to test the possibility. In this study, it was attempted to determine whether CD8$^+$CD28$^-$ Ts also exist in baboon and rat.

MATERIALS AND METHODS

Generation of alloreactive T cell line

Baboon peripheral blood mononuclear cells (PBMC) were separated from buffy coats by Ficoll-Hypaque

centrifugation. Responding PBMC ($4X10^6$/ml) were cultured with irradiated (1600 rad) PBMC ($4X10^6$/ml) from an unrelated baboon donor in complete medium (RPMI 1640 supplemented with 10% fetal bovine serum, 2mM L-glutamine and 50 µg/ml gentamycin) (Gibco, Grand island, NY). After 7 days the responding cells were collected, washed and restimulated with PBMC from the same donor. Recombinant IL-2 (10 u/ml) was added to the culture on day 3 after restimulation. The culture was continued for an additional 4 days before assay. To generate alloreactive T cell line in rat, ACI rats were injected with spleen cells ($50X10^6$) from Wistar-Furth rats on day 0 and 7. The cells were administered IP in balanced salt solution. On day 14, spleen cells were prepared from in vivo primed ACI rats.

Cell separation

$CD4^+$ and $CD8^+$ cell subsets were isolated by negative selection with Dynal CD8 or CD4 magnetic beads (Dynal, Great Neck, NY). The cells ($2X10^7$/ml) primed in vitro or in vivo were incubated with either CD4 or CD8 beads at the concentration recommended by manufacture for 20 minutes at 4^0C. The cell suspensions were then placed on a magnetic plate for 2-3 minutes. The unbound cells were transferred to another tube and washed 3 times. The cells depleted of $CD4^+$ or $CD8^+$ cells ($CD8^+$ or $CD4^+$ T cells) were then suspended in complete medium. To isolate $CD8^+CD28^-$ T cells, $CD8^+$ T cells were further depleted of $CD28^+$ cells with CD28 beads.

Proliferation assays

$CD4^+$ T cells ($4X10^5$/well) were stimulated with irradiated (1600 rad) baboon PBMC or rat spleen cells ($4X10^5$/well) used for priming. $CD8^+CD28^-$ T cells were irradiated (1600 rad) and then added at the initiation of the assay to test their suppressive activity. Cultures were set up in 96-well U-bottom trays in a total volume of 0.3 mL complete medium per well. After 48 hours of incubation, the cultures were pulsed with [^{3}H] thymidine (TdR) and harvested 18 hours later. [^{3}H] TdR incorporation was determined by scintillation spectrometry in an LK Betaplate counter. Mean cpm of triplicate cultures and SD to the mean were calculated. The percent suppression was calculated as [1-(cpm in Th + Ts + APC cultures)/(cpm in Th + APC cultures)] X 100.

RESULTS AND DISCUSSION

To investigate the generality of Ts mediated suppression that we observed in humans, explored was the possibility of generating allospecific Ts in baboon and rat. After two stimulations in vitro with allogeneic PBMC, there is a population of $CD28^-$ T cells in $CD8^+$ subset in baboon T cell line. This population of T cells suppressed the proliferative response of $CD4^+$ T cells from the same T cell line to allogeneic APC in a dose-dependent manner (Fig. 37A). Similarly, rat spleen cells primed in vivo contained a very small population of $CD8^+CD28^-$ T cells. Dose-dependent suppression was also revealed when those cells were added to Th and APC mixture (Fig. 37B).

Peripheral T cell tolerance has been well documented in several experimental animal models of transplantation. However, the cells responsible for and the mechanisms involved in this active regulation remain controversial. It has been recently demonstrated that allograft tolerance induced by different methods such as CD4 and CD8 monoclonal antibody treatment is mediated,

and can be transferred by $CD4^+$, but not $CD8^+$ T cells (5). On the other hand, different groups have also suggested a possible role for $CD8^+$ T cells in tolerance (6). The present data indicate that $CD8^+CD28^-$ subset of T lymphocytes comprises Ts precursors both in rat and baboon. It is possible that failure of $CD8^+$ T cells from tolerant animals to transfer unresponsiveness to secondary hosts may due to heterogeneity of the $CD8^+$ T cell population. The relationship between $CD8^+CD28^-$ T suppressor cells and $CD4^+$ T regulatory cells reported by the other groups is under current study.

References for the Ninth Series of Experiments

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